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UNIVERSITY OF ALBERTA

GENE EXPRESSION IN REGENERATING IMAGINAL DISCS OF *Drosophila melanogaster*

©

by

William John Brook

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Genetics

Edmonton, Alberta
Spring 1994.

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ABSTRACT

In the fruitfly, *Drosophila melanogaster,* imaginal discs are the larval precursors to the adult epidermis. When cultured in an adult female host, complementary disc fragments either regenerate the missing part, or duplicate the remaining part of the normal pattern. The outcome depends on the location of the cut and has been explained by assuming interactions between normally non-adjacent cells which come into contact to form a regeneration blastema when disc fragments heal. This regulative behaviour suggests that the fate of an imaginal disc cell is specified by a positional coordinate system. In order to identify genes which might mediate these events, I screened 826 autosomal insertions of an enhancer-sensitive P-element for altered *lacZ* expression in regenerating discs following cell-death induced by a temperature sensitive cell-lethal mutation, a treatment that causes frequent pattern duplication. The positive insertion lines were sub-divided into four classes based on appropriate timing of the *lacZ* response in relation to the onset of cell-death in the cell lethal mutant, and response γ-irradiation, a different source of cell death. In a direct test using disc fragments cultured *in vivo,* ectopic expression was confirmed in a total of 23 of the lines sampled, and in as many as 50% of the lines which respond to both sources of cell death. In most cases the ectopic expression was spatially and temporally correlated with the formation of the regeneration blastema at the wound heal, and preceded the formation of a duplicate pattern as indicated by ectopic expression of the genes *wingless* and *engrailed.* Based on their normal expression patterns 15 of the 23 lines are not required in imaginal discs during larval development and may represent gene functions recruited from other developmental programs (e.g. control expression in oogenesis or neurogenesis) or the re-iteration of early steps in disc development (expression in embryonic epidermis). Six of the 23 insertions were expressed in control discs at the anterior-posterior compartment boundary, and were only induced in disc fragments when wound healing brought together cells from both compartments. This indicates that some gene expression in regeneration must be dependent on cell-communication, and suggests that the compartment boundaries might play an important role in the process. Three insertions reported genes at four previously characterized loci: *decapentaplegic / transcript-near- decapentaplegic,* which encode a TGF-β homologue and a novel protein, respectively; *crumbs,* which encodes an EGF-repeat protein; and LF06, which encodes a novel protein. The preliminary molecular and genetic characterization of four insertions defining new loci is also reported. For two of these insertions, genomic DNAs were isolated which hybridize *in situ* to imaginal discs in patterns similar to the enhancer-trap expression. Molecular probes for *crumbs* (class I), *dpp, tnd,* LF-06, and G-45 (class II) were ectopically expressed in regenerating imaginal discs, indicating that the enhancer-traps accurately report genuine gene expression. The results indicate that a minimum of 3 % of the sampled insertions are transcriptionally activated early in regeneration and suggest that hundreds of genes may be expressed in the regeneration blastema. The identification, for the first time, of genes expressed specifically in the regeneration blastema when cell fates are being reprogramed by new cell contacts, provides novel material for the analysis of positional specification in this system.

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ABBREVIATIONS

3/4L+EK	Three quarter lateral plus endknob leg imaginal disc fragment
a/p	anterior/posterior
BrdU	Bromodeoxyuridine
cDNA	complementary DNA
CNS	Central nervous system
d/v	dorsal/ventral
DAB	Diaminobenzidene
DIC	Differential interference contrast
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
kB	kilobase
PNS	Peripheral nervous system
RNA	Ribonucleic acid
TTP	Thymidine triphosphate
UM1/4	Upper medial quarter leg imaginal disc fragment
X-gal	5-Bromo, 4-chloro, 3-indolyl galactose
X-phosphate	5-Bromo, 4-chloro, 3-indolyl phosphate

I. INTRODUCTION

Pattern formation is a deceptively simple term used to describe the events controlling the development of uniform groups of embryonic cells into a spatially organized pattern of differentiated cells. The spectacular regularity with which embryonic development proceeds has made it a popular problem for centuries. In the eighteenth century, preformationists argued that an egg must contain a tiny, perfectly formed individual which had only to increase in size; at the time, the opposing philosophy, epigenesis, argued that a vital force acted on the inert material of the egg to direct development (reviewed in Gould, 1977). These ideas are now considered absurd, of course, but the philosophies do reflect two basic mechanisms for pattern formation. Mosaic properties of developing systems were discovered experimentally that suggested a pre-existing distribution of cytoplasmic determinants (reviewed in Slack, 1991). The problem of how these determinants are localized initially is substantial and models of this kind (often called neo-preformationist, Davidson, 1968) had difficulty explaining the regulative properties of some embryonic fields. Cells in regulative embryonic fields were found to act in a coordinated manner, such that removal of some cells in the field was compensated by the remaining cells, leading to a complete embryonic pattern. Because of this, some "epigenetic" mechanism must be held as necessary for development, implying that interaction between the constituents of the egg can give rise to patterns not present initially (Waddington, 1959). In contemporary terms, this is understood as a problem of how the information for pattern encoded in the genome is decoded during development.

The local autonomy of pattern mutants in genetic mosaics led Stern (reviewed in 1968) to postulate that the competence of a cell to differentiate a pattern element was dependent on the ability of its own genome to correctly respond to local singularities, or peaks, in a global pre-pattern. Although the predicted class of non-autonomous pre-pattern mutants were only occasionally found (reviewed in Tokunaga, 1978) and are no longer interpreted in these terms, his ideas laid down some of the framework for the positional information concept. Wolpert (1969) proposed that in a regulative field, cells respond not to singularities in a pre-pattern, but to information about their locations in a globally specified system of positional coordinates. This "positional value" was thought to be locally interpreted by individual cells to determine how the cell will differentiate. Morphogen concentration gradients had been proposed much earlier by Child to explain the properties of several developing and regenerating systems (cited in Wolpert, 1969). Thus Wolpert suggested that one mechanism by which positional information could be specified in developing systems might be by the diffusion of a molecule to create a concentration gradient. The local concentration of this morphogen would be measured by each cell which would develop along the appropriate developmental pathway specified by threshold gradient levels, as put forth in the "French Flag Model".

The reality of such a system of positional information was recently confirmed in the *Drosophila* embryo (Driever et al., 1988), where the concentration of a homeobox DNA binding protein encoded by *bicoid* was shown to control patterning in the anterior posterior axis. However, the general applicability of these findings is uncertain as the gradient forms and is initially interpreted in an acellular system. The *Drosophila* imaginal discs are one of several experimental paradigms for positional information in a cellular system. Although early work held the promise of a genetic model (Hadorn, 1960) very little is known of the genes that might control the specification and interpretation of positional information in this system. The idea that imaginal disc cells are positionally specified is necessitated by the regeneration and duplication behaviour of cultured imaginal disc fragments. This thesis investigates of the genetic response of cells during imaginal disc regeneration and duplication with the goal of identifying some of the genes involved.

I.1 Pattern formation in imaginal discs

I.1.1 Determination of imaginal discs

In Drosophila, the adult cuticle is formed from groups of cells called imaginal discs, which secrete the head, thoracic, and genital cuticle, and abdominal histoblasts which give rise to the abdominal cuticle. The imaginal discs are derived from cells of the embryonic ectoderm which invaginate during embryogenesis to form epithelial sacs evident in the late embryo (Auerbach, 1936; Laugé, 1967). The number of founder cells in imaginal disc primordia has been estimated to range from 2-40 depending on the disc and the method of estimation. These estimates have been derived either indirectly, by analysis of genetic mosaics produced during embryogenesis (for reviews see Hall et al., 1976 and Janning, 1978) or directly, by histological examination of freshly hatched larvae (Madhaven and Schneiderman, 1977) or developing embryos (Bate and Martinez-Arias, 1991).

The cells divide mitotically throughout larval development and begin to differentiate after pupariation. By late third instar, each disc consists of several thousand cells and has a characteristic shape and folding pattern. Mature discs which are dissected from one larva and transplanted into the abdomen of another can differentiate along with the host at metamorphosis (Ephrussi and Beadle, 1936). These experiments show that each imaginal disc gives rise to an invariant set of adult cuticular structures depending on its segmental origin.

The commitment to form these structures was found to occur very early in development. Chan and Gehring (1971) found that cells from the anterior and posterior halves of bisected blastoderm stage embryos were heritably committed to form only anterior and posterior imaginal structures respectively. Simcox and Sang (1983) tested the specificity of these early commitments by transplanting small groups of cells to ectopic positions in host embryos. When transplanted to ectopic sites in the host embryo, marked cells maintained the metameric commitments specified by their original locations in the donor embryos. In many cases, corresponding adult deficiencies were observed in surviving donors. These results are consistent with a developmental commitment to individual metameres as early as the blastoderm.

Also in support of this interpretation are observations of somatic clones induced at blastoderm. With X-ray induced somatic recombination, it is possible to label cells at any stage of development. In this manner, it has been shown that clones labelled at the blastoderm stage are confined to a particular imaginal thoracic segment , although clones which label both the left and right discs occur as do clones which label both dorsal and ventral discs (Steiner, 1976; Wieschaus and Gehring, 1976). These experiments suggest the commitment to form particular segments occurs as early as blastoderm. However, as the results of clonal analysis also suggest, the restriction to discs within a segment must occur later. Meise and Janning (1993) have confirmed this by transplanting single blastoderm cells which express β-galactosidase into wild-type blastoderm hosts. They find that the clonal progeny of these cells give rise to both larval and imaginal cells, thus the restriction to form imaginal structures must occur later than the blastoderm stage. Whitely and colleagues (1991) and Hartenstein and Jan (1992) have shown that the *escargot* gene is expressed exclusively in imaginal cells at 10 hours of development (stage 13 of Campos-Ortega and Hartenstein, 1985). Cohen (1993) has suggested this indicates the latest possible time of the commitment to imaginal development.

The first evidence that restriction of the developmental potential of imaginal disc cells occurs progressively was obtained by analysis of marked somatic clones. Several investigators demonstrated that somatic clones labelled at successive time points in larval development become progressively restricted in their developmental potential (Garcia-Bellido and Merriam, 1971; Garcia-Bellido et al., 1973). These clonal restrictions define what are called compartments. In the wing disc, compartment boundaries form in simple geometric patterns dividing the discs into anterior and posterior, dorsal and ventral, wing and notum, and distal and proximal compartments. Evidence that these compartments may reflect important developmental commitments was suggested by the transformations of the homeotic mutant, *bithorax* (Morata and Garcia-Bellido, 1976) and the mutant *engrailed* (Garcia-Bellido and Santamaria, 1972) which transform domains defined precisely by the compartment boundaries detected by clonal restrictions.

Since discs grow by cell division, any early developmental commitments must be stably inherited through many cell generations before differentiation. Serial transfer *in vivo* culture experiments performed primarily by Ernst Hadorn and his colleagues (a large body of literature reviewed by Hadorn, 1965) showed (except for occasional transdetermination) that the determination of discs to form specific adult segments is indefinitely stable. Schubiger (1969) made a fate map of the male first leg disc by culturing defined fragments. After transplantation into third instar larvae, he found that each fragment gave rise to a particular part of the leg disc pattern. Also, when he extirpated a small group of cells from a disc and transplanted it into a third instar larvae he found that specific pattern elements were missing. Thus, he demonstrated a detailed mosaic of commitments for pattern elements within a disc, perhaps reflecting the successive binary subdivisions into compartments.

I.1.2 Regeneration duplication phenomenon in imaginal discs

Schubiger (1971) also found that although cells in the mature leg disc had highly restricted normal developmental fates, they were not heritably committed to form these structures. When disc fragments were allowed to grow before metamorphosis, either by implanting into an immature host larva, or an adult female followed by a larval host to differentiate, the pattern elements produced often dramatically transcended those specified by the fate map. The effect was also position-specific. Fragments which included the upper-medial-quarter of the disc (UM1/4) could regenerate the entire pattern of the leg disc, while fragments in which the UM1/4 was absent duplicated the pattern specified by the fate map. Thus imaginal disc fragments behave as a regulative field.

Bryant (1971) suggested that the ability of a fragment to regenerate or duplicate was due to a "gradient of developmental capacity" of cells in the imaginal disc. Cells at the cut edge could only regenerate structures specified by a lower level of this gradient. Thus medial fragments would include cells of the highest level of developmental capacity and could regenerate lower lateral pattern elements, but lateral fragments could only duplicate additional lateral pattern elements at levels lower than the cells at the cut edge. In this model, both fragments produce the same structures during pattern regulation (i.e regeneration or duplication) because the cells at the cut edge of each fragment have the same developmental capacity. Similar leg duplications were induced by exposing freshly hatched first instar larvae to 1000 rads of γ-irradiation (Postlethwait and Schneiderman, 1973). The duplications were presumably caused by radiation-induced cell death (Spreij, 1971) and were found to be deficient for medial markers and duplicated for lateral markers. These results supported the medial-high, lateral-low gradient and also implied that the gradient of developmental capacity must be already present in very young discs. This phenomenon was also demonstrated throughout larval development in temperature-sensitive cell-lethal mutant (Russell, 1974).

Bryant (1975) observed that when wing imaginal discs were bisected, one fragment would regenerate and one fragment would duplicate. He observed that a region in the centre of the disc was included in all of the regenerating pieces and in none of the duplicating fragments, implying that if there were a single gradient of developmental potential, as postulated for the leg disc, its high point would be situated in the centre of the wing disc. This model predicted that when separated from the rest of the disc, the centre should regenerate the missing pattern and the remainder should duplicate. This was not the case. The centre duplicated itself and the periphery regenerated the centre. Thus, a simple gradient could not explain these results.

Haynie and Bryant (1976) demonstrated that pattern regulation in wing imaginal discs was due to a process of intercalation, or filling in, of missing tissue. They mixed fragments from opposite edges of the wing disc and found that the fragments could regenerate the intervening structures. When the fragments were cultured alone, they only duplicated. This result suggested that the material regenerated depended on the interaction of cells from different original positions in the disc. A similar result was observed in wing disc fragments from which a 90° (1/4) sector was removed. A model based on the developmental potential of cells at the cut edge predicts that each edge in the remaining 3/4 of the wing disc would act independently, but one pattern, not two as predicted, was regenerated. Scanning electron microscopy of 3/4 wing disc fragments showed that the two cut edges of the disc heal together prior to regeneration (Reinhardt et al., 1977). Thus, cell interactions may instruct the outcome of regeneration.

I.1.3 The polar coordinate model.

A model proposed by French (1975) to explain the regulative behaviour of grafted cockroach legs was applied to explain amphibian limb as well as imaginal disc regeneration/duplication (French et al., 1976; Bryant et al., 1981). The model, suggests cell interactions resulting in intercalation of positional values in two coordinates of "positional information", of the type proposed by Wolpert (1969) to specify fate according to position in the field . One coordinate provided radial information which specified the positional values of cells in the distal to proximal axis (values A through E in Figure 1). The second coordinate was circumferential specifying the angular positional values of cells like the positions of numbers on the face of a clock (hence, the popular name for the polar coordinate model is the "clockface model"). The model explains regulative behavior as the stimulation of cell division caused by the confrontation of cells with different positional values either by grafting of various combinations of limb stumps or by wound healing. It is simple, having only two rules which describe how new positional values are generated in the cells brought together by grafting or healing.

The first is the rule of shortest route of intercalation in the circumferential coordinate. As an example, if the disc is imagined to be the clockface, a 1/4 piece can be removed by cutting along the 12 and 3 radials; upon wound healing of the three quarter piece, cells with the circumferential values 12 and 3 would be confronted, the missing tissue would be intercalated in by the shortest route, filling in values 1 and 2, and thus regenerating the complete pattern. In the 1/4 fragment, cells with values of 12 and 3 would also be confronted, filling in values 1 and 2, leading to a pattern duplication. Thus any fragment which contains more than half of the positional values should regenerate and fragments containing less than half should duplicate. The second, "complete circle" rule states that when a complete set of circumferential values are confronted at a given level in the proximal-distal axis, regeneration of all distal values will occur. This explains the duplication of distal and regeneration of proximal disc fragments. French and colleagues explain regeneration of leg disc fragments which contain roughly one quarter of the circumference, as in the case of the the UM1/4 fragment of Schubiger (1971) described above, by assuming that the positional values are asymmetrically distributed around the disc circumference and that the UM1/4 includes more than half of the positional values as required for regeneration.

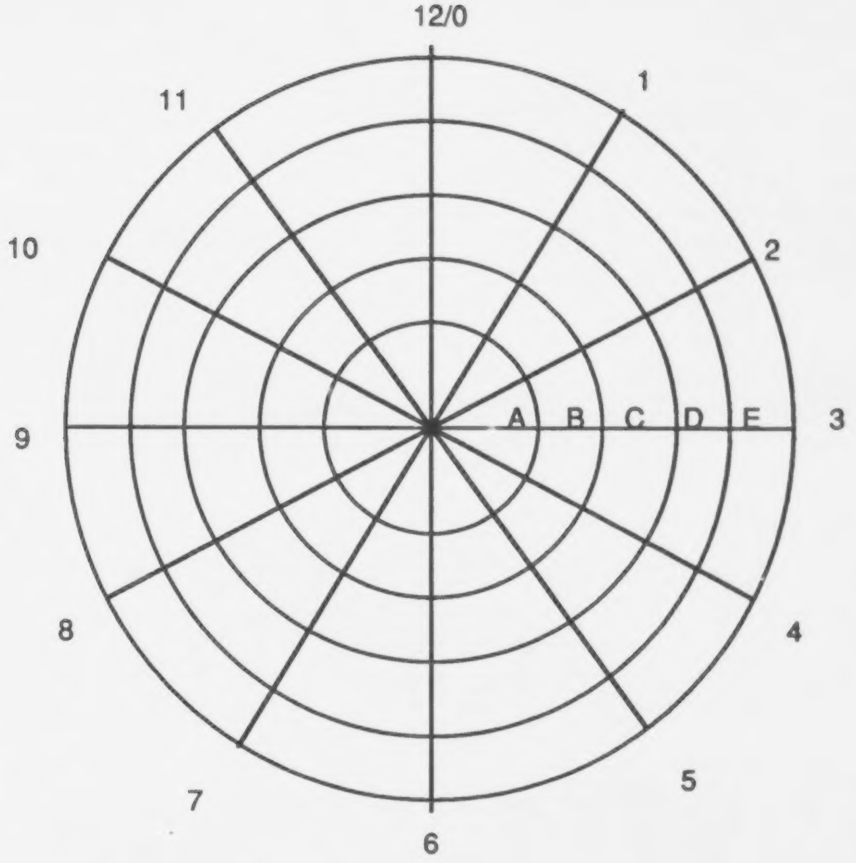
12/0
1
2
3
4
5
6
7
8
9
10
11
A
B
C
D
E

Figure 1: Field map of the polar coordinate model for positional information
(After French et al 1976)

The polar coordinate model is very appealing because it explains the regulative behaviour of three evolutionarily and developmentally distinct systems with one unifying model. It is also appealing because it links growth to pattern formation. According to the polar coordinate model, normal development could proceed by the same intercalation of positional values as is seen in regeneration. Imaginal disc primordia, initiated by a confrontation of the extreme values would grow until a complete spectrum of intermediate positional values were intercalated, although the model does not provide for the specification of the preliminary values. It also provides for the continuous spectrum of positional values in imaginal discs required to explain regeneration and duplication experiments.

I.1.4 Compartments and regeneration

The polar coordinate model ignores the phenomenon of compartmentalization of imaginal discs which is the basis for another theory regarding the specification of cell fates. The selector gene model suggests that each binary compartmental subdivision (i.e anterior vs. posterior, dorsal vs. ventral) is caused by the differential activation of a specific selector gene in one compartment and not in the other (Garcia-Bellido, 1975; Crick and Lawrence, 1975). The progressive bisection of the disc by additional compartmentalization events could be encoded in a binary combinatorial code by the expression of a series of selector genes. Different combinations of selector gene expression would lead to distinctive developmental fates, with the number of selector genes, n, determining the possible number of cell states, 2^n. A few genes with some of the properties expected of compartmental selector genes have been identified by mutations. The *engrailed* gene (*en*) was proposed as the selector gene controlling the anterior versus posterior decision as the posterior compartments of wings homozygous for the mutation *en*^{1} were transformed towards the anterior compartment (Lawrence and Morata, 1975). In this interpretation the activity of the *engrailed* gene would specify posterior compartment. Recently the *apterous* gene (*ap*) has been proposed to act as a dorsal wing selector gene as *ap*$^-$ clones transform dorsal wing tissue to ventral wing (Dias-Benjumea and Cohen, 1993). Both *engrailed* and *apterous* encode homeobox proteins (Laughon and Scott, 1984, Poole et al., 1985, Cohen et al., 1992) which are thought to act as DNA binding transcription factors in conformity with the predictions of the selector gene model. But the digital encoding of positional values envisaged in the selector gene model does not easily explain the continuous variation of positional information in imaginal discs revealed by imaginal disc regeneration.

A question therefore arises as to the role compartments play in the process of regeneration. It is known from studies of regenerating fragments that cells of one compartment can give rise to cells of the other in the regenerated tissue. This is most clearly see in the UM1/4 of Schubiger (1971). This fragment is composed entirely of anterior compartment cells but it is able to regenerate an entire posterior compartment, implying that in this case, the ordinarily cell heritable commitment to an anterior compartment identity is lost in the cells that regenerate the posterior compartment*. Furthermore, during regeneration or duplication in wing (Szabad et al., 1979) or leg discs (Girton and Russell, 1981; Abbott et al., 1981) it has been demonstrated that clones induced prior to wounding can cross anterior/posterior compartment boundaries in the regenerated or duplicated limb, but clones which are induced as early as one day following wounding are again compartmentally restricted. This new clonal restriction is established before regenerative cell divisions (intercalation) are initiated. This implies that the cells which give rise to the new pattern, termed the regeneration blastema (see section I.1.6) lose otherwise stable compartmental commitments but reform them very early in the process of pattern regulation, suggesting that an A/P confrontation might be an essential precondition for pattern regulation. This may also be interpreted as a re-iteration in regeneration of one of the earliest steps in imaginal disc development because the A/P compartment boundary is first established in the extended germ band embryo when stripes of *engrailed* expression establish parasegment boundaries (Martinez-Arias and Lawrence, 1985; Vincent and O'Farrell, 1992).

Compartments and compartment boundaries have also been implicated in distal regeneration from proximal fragments. Schubiger and Schubiger (1978) studied distal regeneration of proximal first leg fragments which included various fractions of the proximal circumference. In contravention of the complete circle rule of French et al. (1976) they found that distal outgrowth occurred quite frequently in fragments which according to the polar coordinate model had no more than 4 or 5 of the 12 "clockface" values and which also duplicated proximal markers. However, as noted by Meinhardt (1983), the ability of these fragments to regenerate distal structures at a high frequency was associated with the number of compartments they contained. In the leg discs there is tentative evidence for a dorsal ventral subdivision of the anterior compartment (Steiner, 1976). Duplicating fragments which contained cells from all three putative compartments were able to regenerate distally at a frequency of 88 %, while a series of four fragments containing posterior compartment tissue and only one of the anterior compartments regenerated distally only 0%, 16%, 19% or 25% of the time, respectively. This suggests that the ability of fragments to regenerate distally may depend on the cooperation between cells of different compartments. The ability of the UM1/4 to completely regenerate remains unexplained in this context in that it is comprised of only anterior compartment cells. However, it should be noted that all distal regeneration in UM1/4 fragments was accompanied by at least partial regeneration of the posterior compartment.

A further example of the possible role of compartments in distal regeneration in leg imaginal discs come from the observation of pattern

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*The terms medial and lateral refer to the orientation of the disc within the larvae. Anterior/Posterior refers to the orientation of the adult structures produced by the disc. In the leg disc, medial corresponds to anterior compartment and lateral corresponds to posterior compartment.

triplications induced with the temperature sensitive cell lethal mutant of *su(f)* (Russell, 1974). Pattern triplications have three copies of some pattern elements following pattern regulation. One ectopic copy always has opposite handedness to that of the original while the second copy has the same handedness. Both ectopic copies arise from a single symmetric base pattern and either diverge to form two distally complete patterns or converge to form two distally incomplete patterns. Girton (1981) observed that pattern triplications in the tarsal segments which contained cells from all three compartments at the base were distally complete. Conversely all triplications which encompassed only two compartments at the base were distally incomplete. It should be noted that the presumed location of the dorsal ventral compartment boundary is only extrapolated to the tarsal segments based on its location in the more proximal segments. These results suggest that the compartments might cooperate to promote distal regeneration (Meinhardt, 1983).

Analogous results have been described in the wing disc by Karlsson (1980). She found that proximal wing disc fragments which contain only anterior or posterior tissue could never regenerate distal structures. Fragments including both compartments could often regenerate distal structures, sometimes even in the absence of circumferential regeneration. It was also seen that the distal regeneration depended on the presence of the ventral a/p boundary, but fragments containing only the dorsal a/p boundary failed to regenerate distally. In one set, fragments which were intended to contain only anterior tissue but with one cut close to the anterior posterior compartment boundary, there was a perfect correspondence between distal regeneration and the accidental inclusion of a posterior compartment marker near the ventral a/p boundary, presumably as a result of an inaccurate cut.

These results all contradict the "complete circle rule" of French et al. (1976) because substantial distal regeneration occurs in the absence of a complete set of circumferential values. A modified version of the polar coordinate model (Bryant et al., 1981) can account for the ability of disc fragments and limb grafts with incomplete circumferential values to regenerate distally. But, the newer model does not explain (or even address) the association of distal regeneration with compartments. Karlsson (1981) attempted to map the distribution of circumferential values assumed by the polar coordinate model onto the wing disc fate map. If a regenerating fragment must contain at least half the positional values any fragment that predominantly regenerates should contain at least 7 clockface values. A fragment that predominantly duplicates should contain 5 or less, and fragments which sometimes do both appreciably should represent transition points where the cut produces two fragments with 6 values. Inaccuracy of cutting could easily explain the indeterminacy of the behaviour of these fragments. Using a series of 10 cuts she was able to resolve the distribution of the positional values required. She estimated that 7-8 of the twelve clockface values must be clustered at the two ends of the a/p boundary. This raises the possibility that compartment boundaries act as "sources" of gradients of positional information.

I.1.5 The boundary model

Meinhardt (1983) proposed a model for positional information in imaginal discs based on the apparent association of compartment boundaries and positional information. He proposed that the point where the anterior-dorsal, anterior-ventral, and posterior compartment come together, is a singularity which acts as a source of a morphogen gradient to specify positional information in the proximal distal axis (see Figure 2). He proposes a similar gradient origin to form in the wing disc at the intersection of the a/p and d/v boundaries. Thus the observed requirement for cells from more than one compartment to get distal regeneration, would be due to the requirement for cooperation between compartments to establish the high point of the gradient.

Meinhardt's boundary model is similar to the polar coordinate model in terms of circumferential positional information. He proposes that compartment boundaries act as cardinal positional values and that the intermediate positional values are subsequently intercalated. He also proposes that fragments regenerate if all of the compartmental states are included and that fragments which are missing a compartmental state will duplicate. The regeneration of fragments like the UM1/4 leg disc fragment which has no posterior cells, is arbitrarily explained by the respecification of some cells to the missing compartmental state. How this respecification could occur is not satisfactorily explained.

The polar coordinate model and the boundary model share certain features. This is probably because they both attempt to explain the same results from different perspectives. The polar coordinate model accounts for the regulative behavior of the disc field assuming the intercalation of an existing continuum of positional values, while the boundary model describes a process that could generate this system of positional information. The boundary model is in essence a version of the polar coordinate model which attempts an integration of the selector gene and positional information theories of pattern specification, but certain results are still not convincingly explained by the synthesis.

I.1.6 Cellular dynamics of imaginal disc regeneration

The preceding sections describe the pattern transformations that occur in disc fragments after cell division during *in vivo* culture. Several studies have also addressed the cellular processes involved. Transmission electron microscopy ultrastuctural studies of wing imaginal discs during regeneration have shown that wound healing of the cut edges produced during fragmentation proceeds though several stages (Reinhardt and Bryant, 1981). Imaginal discs are sac-like structures which have two distinct epithelial morphologies, the "disc epithelium proper" which has a columnar arrangement and which eventually secretes the adult cuticle, and the peripodial membrane, a squamous epithelium which is thought not to contribute to the adult fate map. The initial events in regeneration include a thickening of the peripodial cells and a heterotypic interaction between the disc epithelium and peripodial membrane along the edges of the wound. As the edges of the wound heal

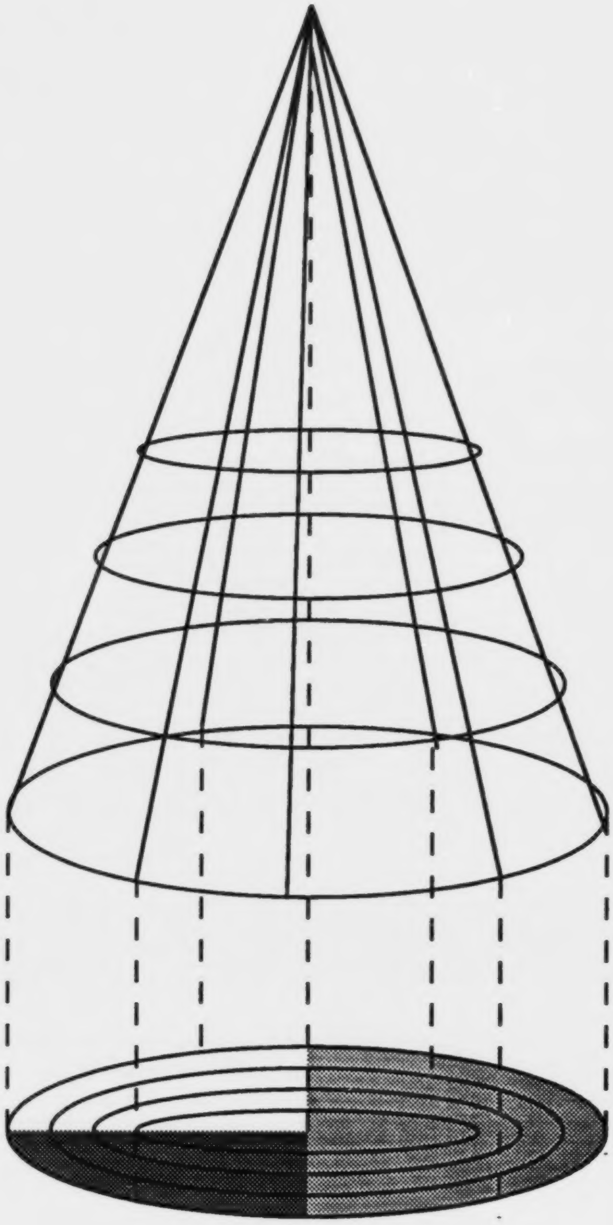


Figure 2: Boundary model for imaginal disc positional information
(After Meinhardt (1983))

together, the heterotypic interactions cease and the disc epithelia and peripodial membranes heal to restore the continuity of the cell layers.

Disc cells exhibit atypical behaviours during disc regeneration including the formation of pseudopodial processes during the re-establishment of cell-cell contacts and unusual cell shape changes. There is also some cell death in the vicinity of the cut edge, possibly caused by surgical damage. Scanning EM pictures of the wound during healing reveals it to be covered with cellular debris and occasionally macrophage-like cells, which may be haemocytes, possibly scavenging debris at the damaged cut edge (Reinhardt et al., 1977).

Clonal analysis of regenerating or duplicating leg discs show that a relatively small number of cells participates in regeneration. Girton and Russell (1980) estimated that the average founder cell population of duplicated limbs induced with the temperature sensitive cell lethal to be about seven cells. It is of interest that this is similar to the estimates of the number of cells in imaginal disc primordia described earlier. Abbott and colleagues (1981) showed that clones induced in leg discs at the time of cutting, extended into the duplicate or regenerate, but were restricted to structures near the site of the wound in the original part of the leg, suggesting that the cells taking part in regeneration come from sites close to the cut edges. This is consistent with the results from many studies where labelling of fragments either with tritiated TTP or with Bromodeoxyuridine (BrdU) has been used to map the distribution of S-phase cells (Dale and Bownes,1980; Kiehle and Schubiger, 1985; O'Brochta and Bryant, 1987; Bryant and Fraser, 1988). In general these studies show that S-phase cells are found above background levels only within 4 or 5 cell diameters of the wound.

Dale and Bownes (1980) looked at whole mounts of imaginal wing disc fragments and found that incorporation of label was most intense at the site of wounding. Kiehle and Schubiger (1985) confirmed this observation by showing that the proportion of labelled cells near the cut edges of leg disc fragments was higher than in the remainder of the disc after 1 day in culture. Schubiger and Karpen (1983) have pointed out that the higher mitotic activity and regenerative ability of the cells at the cut edge are also characteristics of regeneration blastema cells in amphibian limb stumps (Goss 1969). The amphibian blastema is defined also on histological criteria such as cell de-differentiation and the formation of a wound epithelium covering the stump. In disc fragments, the blastema consists of the dividing population of cells which gives rise to the regenerate or duplicate. Further evidence for the special nature of these cells comes from experiments where the blastema is removed from the remainder of the disc after one day in culture. Under these conditions, it can can greatly exceed its normal potential. It has been shown that normally duplicating blastemas can regenerate the entire disc pattern when separated from the remainder of the disc (Karpen and Schubiger, 1981). This is not predicted or easily explained by either the polar coordinate model or the boundary model.

O'Brochta and Bryant (1987) have demonstrated that the size of the blastema in wing disc fragments is proportional to the number of cells at the cut

Table 1: Summary of the characteristics reported for the cells of the imaginal disc regeneration blastema.

Phenomenon	**Study**
Atypical cell morphologies and cell contacts	Reinhardt et al (1977), Reinhardt and Bryant (1981)
Increased S-phase labelling and mitosis	Dale and Bownes (1980), Kiehle and Schubiger (1885), O'Brochta and Bryant (1987), Bryant and Fraser (1988)
Respecification of compartmental commitments	Szabad et al (1979), Girton and Russell 1981, Abbott et al., (1981)
Extensive capability for regeneration	Karpen and Schubiger (1981), Schubiger and Karpen (1983)

edges of the wound and that it persists throughout regeneration. Various cuts produced blastemas with average numbers of labelled S-phase cells ranging from 150-450. When discs were cut but no tissue was excised, the ability to incorporate label was lost from 85% of the fragments within 3 days, but when large fragments of tissue were excised, between 35-100 % of fragments still contained a blastema after 5 days of culture. The difference in blastema size as estimated by clonal analysis (Girton and Russell, 1980) may have to do with the size of the wound which induces pattern regulation in the two situations. However, it is also possible that only a sub-set of the labelled cells actually comprise the blastema.

Bryant and Fraser (1988) examined the appearance of S-phase cells in relation to actual growth of regenerating imaginal disc fragments and found that BrdU incorporation preceded wound healing, but did not correlate to the initiation of growth, implying that cells were proceeding through S-phase and arresting in G2. Growth only occurred following wound healing. A similar phenomenon was seen in dissociated, reaggregated imaginal discs cultured *in vivo* (Fain and Alvarez, 1987). It was found that the proportion of G2 cells increased sharply in the first 8 hours of culture and then decreased as the cell number grew, implying a similar G2 block and release.

I.2 Genetics of Imaginal Disc Pattern Formation

Shearn and colleagues (1971) attempted to identify mutations in genes controlling imaginal disc development by screening for third chromosome pupal lethal mutations. These and subsequent screens (Shearn et al., 1974; Kiss et al., 1976) have identified literally hundreds of mutants which have small discs or lack discs altogether. Some of these mutants caused homeotic transformations in specific discs, and others caused disc overgrowth phenotypes, but in general these mutants probably do not define many genes involved in disc pattern formation and/or the interpretation of positional information. Indeed, a survey of these mutations has demonstrated that perhaps as much as half of them cause cell cycle defects, suggesting that the small disc and discless phenotypes may be due to inability of the disc cells to divide mitotically (Gatti and Baker, 1989).

On the other hand, saturation screens for mutations affecting the embryonic cuticular pattern and the subsequent developmental and molecular analysis of these genes have led, in the period of just over a decade, to a detailed understanding of the molecular basis of positional information and pattern formation during Drosophila embryogenesis (for reviews see Ingham, 1988; St. Johnston and Nüsslein-Volhard, 1992). Coupled with molecular-genetic characterization of the two major homeotic complexes in Drosophila (Duncan, 1987; Mahaffey and Kaufman, 1988), a coherent view of the specification of pattern in the larval epidermis is now possible. It is generally accepted that the specification of the anterior posterior pattern in the embryo is controlled by a hierarchy of regulatory proteins which subdivide the embryo into progressively smaller repeat units (metameres) which are distinguished from each other by the differential expression of the homeotic selector genes of the Antennapedia and bithorax complexes.

The primary specification of anterior posterior pattern is controlled by *bicoid*, which encodes a homeodomain DNA binding protein (Driever et al., 1988). Maternal *bicoid* RNA is localized at the anterior pole of the oocyte and the protein product diffuses through the syncitial pre-blastoderm embryo to form an A/P-concentration gradient. The embryo is then subdivided into a series of contiguous regions by the gap genes, which are directly, or indirectly, transcriptionally regulated by bicoid gradient concentration (reviewed by Pankratz and Jäckle, 1990). These genes also encode regulatory DNA binding proteins. One group of gap genes regulates the development of the head segments directly (Finkelzstein and Perrimon, 1990; Cohen and Jurgens, 1990, 1991) and the second group regulates a second tier of zygotic transcription factors, the pair-rule genes, which divide the thoracic and abdominal fate map into two-segment units. The "head gap" and the pair-rule genes specify the expression of the segment polarity genes. The segment polarity genes encode transcription factors and proteins which appear to be involved in cell-cell signalling (Hooper and Scott, 1992).

The initial specification of the dorsal ventral pattern is also determined by a gradient of a maternally contributed DNA binding protein, encode by *dorsal*. A dorsal-low, ventral-high gradient of the nuclear localization of this transcription factor is formed in the blastoderm embryo. Thus both major embryonic axes in the Drosophila embryo are controlled by gradients of morphogens. These results validate the predictions of Wolpert (1969) in that cells in the Drosophila blastoderm could have positional information specified on Cartesian coordinates. However, the syncitial blastoderm stage, where the specification and interpretation of this positional information occurs, is an acellular system so the general applicability of these findings remains to be investigated. Both the bicoid and dorsal morphogens are transcription factors. It is hard to imagine how such molecules could function as diffusible morphogens in a cellular system.

Several lines of evidence link segmental patterning to pattern formation in the imaginal discs.Embryonic lethal mutations in the genes at the final level of the anterior posterior segmentation gene hierarchy, the segment polarity genes, yield phenotypes which involve deletion of part of the segment and mirror image duplication of the remainder (Nüsslein-Volhard and Wieshcaus, 1980). This is formally similar to the pattern transformations found in other insect species after more than one half of a post-embryonic segment is excised (Locke, 1959; Wright and Lawrence, 1981) and in duplicating disc fragments. This similarity has been noted in several models suggesting a common basis for positional information in insect segments and limbs/imaginal discs (Meinhardt, 1983; Meinhardt, 1986; Russell, 1985; Martinez-Arias, 1989). The segment polarity gene *wingless* is required for the allocation of imaginal disc primordia from the embryonic ectoderm (Simcox et al., 1989; Cohen et al., 1993). It has also been demonstrated that when lethal embryos homozygous for either of the segment polarity mutants *patched* and *naked* are cultured *in vivo* disc primordia can be rescued. The pattern duplications detected in these discs by staining for *engrailed* expression correspond to the specific segment polarity phenotypes of those mutants (Simcox et al., 1989; Cohen, 1990). Finally, many of the segment polarity genes have adult viable alleles which cause pattern transformations (reviewed by Wilkins and Gubb, 1991) or position specific pattern defects in adult limbs (Held, 1993).

I.3 Strategy for genetic analysis of imaginal disc regeneration

As the review of the literature suggests there is a gap in our understanding of pattern formation and pattern regulation in imaginal discs. While regeneration and duplication indicate a positional coordinate system of some nature, very little is known about the genetic basis of these processes, especially in comparison to the breadth of understanding of positional specification in the embryo. This is important as patterning in imaginal discs is elaborated in a cellular system which is more representative of developing systems in animal phyla than the acellular syncitial blastoderm.

This thesis presents an attempt to identify genes expressed during imaginal disc regeneration using the enhancer trap method of O'Kane and Gehring (1987). O'Kane and Gehring devised a P-element construct which carries an *E. coli lacZ* reporter gene driven by the P-transposase promoter. The promoter is very weak and expression of the β-galctosidase protein is only detectable when the P-element is inserted next to genomic regulatory sequences. The approach is very sensitive to position specific enhancers as the majority of the original sample of 49 transformants selected by O'Kane had patterned *lacZ* expression.

Large collections of enhancer-traps have been generated by remobilizing transformed constructs with the stable genomic transposase source P[Δ2-3; *ry*$^+$] (99B) (Robertson et al., 1988). The enhancer constructs employed in these screens include bacterial origins of replication and antibiotic resistance markers to allow cloning of flanking genomic DNA by plasmid rescue (Bier et al., 1989; Bellen et al., 1989). There are several examples of faithful reporting of transcription patterns by enhancer-trap insertions near genes whose expression is well known (for examples see Wilson et al., 1989). The screens have been used to generate markers for development (Bellen et al., 1989; Perrimon et al., 1991; Hartenstein and Jan, 1992), to study specific tissue types (Bier et al., 1989; Mlodzik et al., 1990; Doe et al., 1991), or to identify targets of homeotic genes (Wagner-Bernholz et al., 1991).

In this study, enhancer-traps were used to identify for the first time, a set of genes expressed in regenerating imaginal discs. This approach was chosen because the enhancer-trap *lacZ* expression may be screened as a dominant, thus insertions which cause early lethality can still be recovered and tested for expression during regeneration. This is critical as disc regeneration may re-iterate mechanisms essential for pattern formation in early development. These functions would be substantially more difficult to detect in a conventional phenotypic screen. Furthermore, the enhancer-trap provides convenient starting material for genetic and molecular characterization of flanking genes, as well as information about the expression of putative regeneration genes in other developmental processes.

Chapter III describes the screening of enhancer-traps in a temperature sensitive cell-lethal background. Sublethal temperature pulses in the mutant *su(f)*^{12} lead to imaginal disc cell-death and pattern duplications at a high frequency (Russell, 1974; Russell et al., 1977; Clark and Russell, 1977; Girton and Kumor, 1985). This circumvents the practical difficulty of screening large numbers of insertions by culturing disc fragments. A selected sample of positives from the cell-lethal screen was also shown to be expressed in cultured disc fragments. These results have been published previously (Brook et al., 1993).

Chapter IV presents the identification of three enhancer-traps expressed during regeneration at previously described loci, as well as the preliminary molecular and genetic characterization of four insertions at novel loci. Finally, the dynamics of expression of several insertions relative to the disc pattern genes *wingless* and *engrailed* are compared in a regeneration time course in Chapter V. The results show that the screen identifies extensive ectopic gene expression in regenerating cells preceding redeployment of *wingless* and *engrailed* expression leading to pattern regulation.

II. MATERIALS AND METHODS

II.1 Drosophila culture

Drosophila cultures were reared on standard medium (10 g agar, 100 g sucrose, 100 g brewer's yeast, 100 mg chloramphenicol, 4.3 g sodium phosphate dibasic, 2.7 g sodium phosphate monobasic, 10 ml propionic acid, distilled water per litre, (pH 7.4)) (Nash and Bell, 1968). Fly stocks and crosses were kept at room temperature (20°C - 22°C) or in a 25°C incubator, unless otherwise indicated.

II.2 Drosophila Strains

Unless specified otherwise, a full description of all the following markers and balancers can be found in "The genome of Drosophila melanogaster" (Lindsley and Zimm, 1992).

Drosophila strains used in this study

Stock	Source
1. ***PZ[lacZ ry^{+}]; +; ry^{506}***	Amanda Pickup, Dept. of Biology, University of California, Los Angeles.
2. ***pr cn;T(2;3)CyO-TM6A; mwh ry e***	J. Merriam , Dept. of Biology, University of California, Los Angeles.
3. ***w; Sb ry e P[Δ2-3,ry^{+}]99B / TM6A, Ubx e***	J. Merriam , Dept. of Biology, University of California, Los Angeles.
4. ***y v f su(f)^{12}***	M.A. Russell, Dept. of Genetics, University of Alberta
5. ***Canton-S***	M.A. Russell, Dept. of Genetics, University of Alberta
6. ***Df(3R)crb$^{S87\text{-}4}$ / TM1, Mé e***	Ullrich Tepaß, Dept. of Biology, University of California, Los Angeles.
7. ***crb^{MA22} rucuca / TM3 Sb e***	Ullrich Tepaß, Dept. of Biology, University of California, Los Angeles.
8. ***Df(2R)nap^{8} / In(2LR)Gla***	R. Kreber, University of Wisconsin, Madison.
9. ***Df(2R)nap^{9}, cn bw / In(2LR)Gla***	R. Kreber, University of Wisconsin, Madison.
10. ***Df(2R)nap^{11} / In(2LR)Gla***	R. Kreber, University of Wisconsin, Madison.
11. ***In(2R)bw^{VDE2LCy2R} / In(2LR)Gla***	R. Kreber, University of Wisconsin, Madison.

12. *Df(3L)Cat*^{DH104}*, rl red / TM6B*	Anita Matthews, University of North Carolina
13. *Df(3L)Wr4, ru h e ca / TM6B*	M. Abbott, Kansas State University
14. *Df(3l)Wr10, ru sbd e /TM6B*	M. Abbott, Kansas State University
15. *GR844*^{X22}*/CxD*	Kristen White, MIT
16. *GR844*^{X31}*/ TM3, Sb e*	Kristen White, MIT
17. *GR844*^{X33}*/ TM3, Sb e*	Kristen White, MIT
18. *mld cn bw sp / CyO*	Kathy Matthews, Indiana University, Drososphila stock centre.
19. *bw; Df(3R)fat*^{bP}*, st / TM6B*	J. Fisher-Vize, University of Texas Austin.
20. *ry Sb P[Δ2-3,* ry^{+}*](99B) / TM2, Ubx P[Δ2-3,* ry^{+}*](99B)*	Tim Heslip, University of Alberta
21. *Sp / CyO; ry Sb P[Δ2-3,* ry^{+}*](99B) / TM6, Ubx e*	Tim Heslip, University of Alberta
22. *Df(3L)CatDH104, Sb e P[Δ2-3,* ry^{+}*](99B) / TM6B*	This study
23. *Sp / CyO; ry*	Tim Heslip, University of Alberta
24. *CyO; T(2;3)Xa, ap*^{Xa}*; TM2, red ry Ubx e*	M.A. Russell, university of Alberta
25. *TM3, Sb ry e / TM6, Tb Hu e*	Kathy Matthews, Indiana University, Drososphila stock centre.
26. *TM3, Sb e / TM6, Tb Hu e*	M.A. Russell, University of Alberta .
27. *cn; T(2;3)CyO-Tb*^{ch}*; TM3, Sb e*	Stanley Tiong, University of Alberta
28. *S Sp Tft nw*^{B} *Pin*^{Yt} */ CyO*	John Locke, University of Alberta
29. *Df(2L)Gdh-α / CyO*	Debra Long, University of Alberta
30. *Df(2L)cl*^{h1}/ CyO	Debra Long, University of Alberta
31. *Df(2L)cl*^{h2}/ CyO	Debra Long, University of Alberta
32. *Df(2L)cl*^{h4}*/CyO*	Debra Long, University of Alberta
33. *Df(2L)E66/CyO*	Debra Long, University of Alberta
34. *Df(2L)cl*^{1}*/CyO*	Debra Long, University of Alberta
35. *Df(2L)cl*^{7}*/CyO*	Debra Long, University of Alberta
36. *wg-lacZ / CyO*	A. Manoukian, University of Toronto.

37. *Tn(2)BS3.0, cn;* *ry*^{506} R. Blackman, University of Illinois, Urbana

II.3 Bacterial stocks

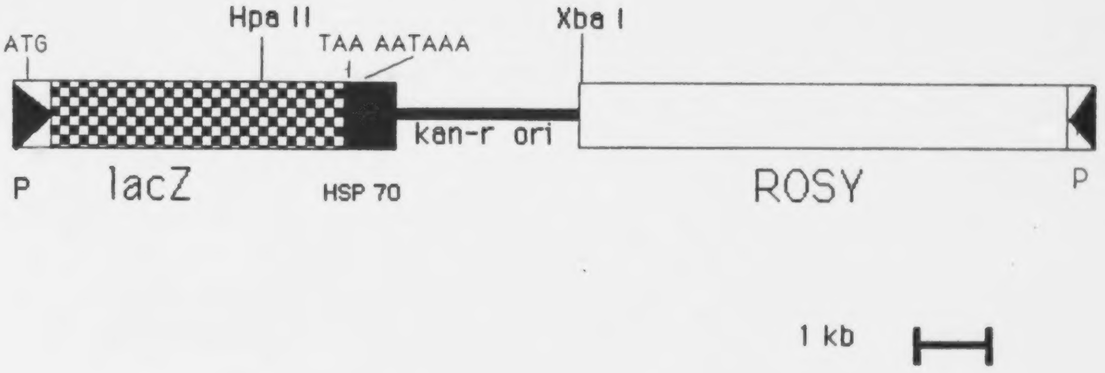
DH5α (endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA)U169 ϕ80dlacZΔM15) was received from Mr Andrew Simmonds and Ms. Laura Querengesser, University of Alberta.

II.4 Generation of Enhancer-Trap Insertions

The enhancer-trap used in this study was the PZ element (Jacobs et al., 1989; Dr. Y. Hiromi, pers. comm.). Figure 3 is a diagram of the PZ element. A more complete restriction map appears in Figure 19.

Figure 4 shows the scheme used to generate insertions. Single males carrying an X-linked insertion of the PZ enhancer-trap construct (Jacobs et al., 1989) and the Δ2-3 transposon, which is a constitutive source of P-transposase (Robertson et al., 1988), were crossed to females of the genotype *pr cn; T(2;3)CyO-TM6; mwh ry e*. Cy$^+$, Ubx$^+$ male progeny carrying the *rosy*$^+$ marker of the enhancer-trap were considered to contain an autosomal transposant because of the male parent to male offspring transmission of the X-linked *rosy*$^+$ marker. These males were then mated to *pr cn; T(2-3)CyO-TM6; mwh ry e* females. The Cy , Ubx offspring from this cross were stocked and the Cy$^+$, Ubx$^+$ offspring scored for the *pr* (purple eyes), *cn* (cinnabar eyes), *ry* (rosy eyes) and *e* (ebony body colour) markers. Lines in which *rosy*$^+$ segregated from *pr* and *cn* were classified as second chromosome insertions, lines in which *rosy*$^+$ segregated from *e* were classified as third chromosome insertions, and lines in which *rosy*$^+$ segregated from none of these markers were classified as fourth chromosome insertions. In some cases, lines originally designated as fourth chromosome inserts were later found to have two insertions: one on chromosome two and one on chromosome three. Lines where no Cy$^+$, Ubx$^+$ progeny were detected in the stocked progeny were tentatively classified as lethal insertions pending reversion tests to exclude coincidental second site lethals. Many lethal insertions, which were initially not classified with respect to linkage were mapped later in the course of determining their lethal stages and / or phenotypes, thus a higher proportion of the lethal insertions were assigned linkage.

Figure 3: Diagram of the PZ enhancer-trap element used in this study (Jacobs et al., 1989). Shown on the diagram are the P-element ends (black triangles), the translation start site within the left end (ATG), the *E.coli lacZ* reporter gene (checkered box) the *D.melanogaster* HSP 70 terminator sequences (black box) containing the stop codon (TAA) and poly-A (AATAAA) sites , plasmid sequences (black line) containing the the kanamycin resistance marker (kan-r) and bacterial origin of replication required for plasmid rescue, and the *rosy*$^+$ marker which indicates the presence of the transposon in fly crossing schemes. The location of unique *Xba* I and *Hpa* II restriction sites which allow the cloning of left and right flanking sequences, respectively, are also indicated.

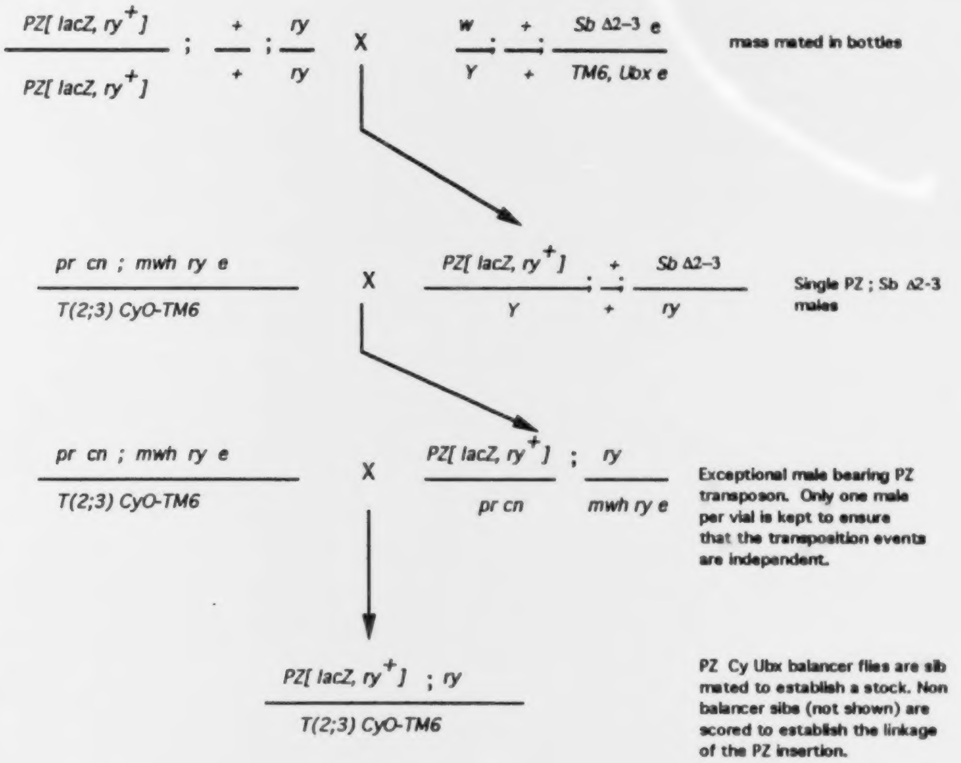

Hpa II
Xba I
ATG
TAA AATAAA
kan-r ori
P
lacZ
HSP 70
ROSY
P
1 kb

PZ

Figure 4a: Crossing scheme to generate autosomal enhancer-trap insertions (After Cooley, Kelly, and Spradling 1988). The figure represents the recovery of a second chromosome insertion. Females homozygous for an X-linked PZ insertion were crossed to males carrying the Δ2-3 element, a stable P-element which produces P-transposase a high constitutive levels, on a third chromosome carrying the dominant marker, *Stubble (Sb)*. Progeny males carrying both the PZ and the Δ2-3 elements were crossed to females from a marked balancer stock, *CyO-TM6*. The majority of the Cy$^+$, Ubx$^+$ males from this cross are rosy. Exceptional rosy$^+$ males arise from the transmission of a PZ element from male parents to male offspring. This usually results from the inheritance of a PZ element that has transposed from the X-chromsome to an autosomal location. Exceptional males were crossed to females from the *CyO-TM6* stock. The progeny from this stock served two purposes: The Cy Ubx flies were mated to establish a stock and the Cy$^+$ Ubx$^+$ flies were scored to determine the linkage of the PZ element. The *ry*$^+$ marker of the PZ element segregated from pr cn for chromosome II insertions, from the e chromosome for chromosome III insertions, and assorted independently of both the pr cn and e chromosomes for chromosome IV insertions. The progeny of the Cy Ubx sib mating were kept as a stock. If no Cy$^+$ Ubx$^+$ progeny were seen, the stock was designated as a lethal insertion.

Figure 4b: Summary of linkage and homozygous lethality of enhancer-trap insertions.

a)


PZ[lacZ, ry+] ; + ; ry
PZ[lacZ, ry+] ; + ; ry
X
w ; + ; Sb Δ2–3 e
Y ; + ; TM6, Ubx e
pr cn ; mwh ry e
T(2;3) CyO-TM6
X
PZ[lacZ, ry+] ; + ; Sb Δ2–3
Y ; + ; ry
pr cn ; mwh ry e
T(2;3) CyO-TM6
X
PZ[lacZ, ry+] ; ry
pr cn ; mwh ry e
PZ[lacZ, ry+] ; ry
T(2;3) CyO-TM6

mass mated in bottles

Single PZ ; Sb Δ2-3 males

Exceptional male bearing PZ transposon. Only one male per vial is kept to ensure that the transposition events are independent.

PZ Cy Ubx balancer flies are sib mated to establish a stock. Non balancer sibs (not shown) are scored to establish the linkage of the PZ insertion.

b)

chromosome		two	three	four	unmapped
total	826	155	198	10	463
lethal	139	45	54	nd	40
	(.17)	(.29)	(.30)	(nd)	(.08)

The enhancer trap lines generated for this study were assigned letters and numbers which correspond to the order in which they were generated. The first one hundred were numbered A-00 to A-99, the second hundred were numbered B-00 to B-99, and so on. Thirteen strains carrying the original O'Kane and Gehring (1987) enhancer-trap were obtained from Dr. J. Merriam, UCLA. These lines have two-letter designations (e.g. AD-55).

II.5 Lethal stages and phenotypes of homozygous lethal insertions

Embryonic lethality of enhancer-trap insertions was determined as described in Wieschaus and Nüsslein-Volhard (1980). Balanced insertion-strain males were crossed to Canton-S virgins and F1 non-balancer sibs (5-10 of each sex) were mated and allowed to lay eggs for 24 hours on yeast-agar medium in 60 mm petri dishes. The eggs were counted and allowed to develop for 24 hours. Unhatched eggs were dechorionated on double sided sticky tape and the total number of dead embryos and unfertilized eggs was determined by examination with a dissecting microscope prior to mounting. If roughly 1/4 of the fertilized eggs died as embryos an insertion was considered to be homozygous embryonic lethal and all the dead embryos were devitellinized with fine forceps and mounted in (9:1) lactic acid : 95% ethanol under a coverslip and cleared overnight at 45°C. The cleared cuticle preparations were then scored for morphological abnormalities.

Larval and pupal lethality was determined using the dominant marker *Tubby (Tb)*. Briefly, second and third chromosome lethal insertions balanced by *T(2;3)CyO-TM6* were crossed to stocks containing *T(2;3)CyO-Tb*^{ch} or *TM6B, Tb* balancers respectively. The F1 Tb, non-*CyO-TM6* progeny were mated and allowed to lay eggs for 24 hours on yeast-agar medium in petri dishes. After incubation, the resulting larvae were scored daily for the presence of Tb$^+$ larvae homozygous for the insertion chromosome. In cases, where Tb$^+$ pupae did not appear, the last stage when Tb$^+$ larvae were seen was considered to be the lethal stage. When uneclosed Tb$^+$ pupae were found, the pupal cases were dissected and the pupae scored as either pupal lethal if there was no apparent differentiation or if there was some differentiation of adult cuticle, or pharate adult lethal if there was considerable differentiation of imaginal structures.

II.6 γ-Irradiation

γ-irradiation of larvae was preformed using a Gamma cell 220 ^{60}Co γ–ray source which discharged at a rate of approximately 450 rads/min at the time of this study. To induce cell death in imaginal discs, doses of 2500 rads (~5.5 minutes) were commonly used as this treatment causes extensive imaginal disc cell death (James and Bryant 1981).

II.7 Imaginal disc *in vivo* culture

Disc *in vivo* culture was performed skillfully by Ms. L.M. Ostafichuk, essentially as described by Ursprung (1967). Needles for injection were made by pulling 25 µl "Microcaps" capillary tubes (Drummond Scientific Co.) over an alcohol burner by hand. The tips were broken in order to give them a bevelled edge of the desired diameter (approximately the diameter of the imaginal disc fragments to be injected). This was determined empirically by comparison to dissected discs. The bevelled edge was sharpened on Saphine Film (Circon Corp.). Needles were attached to a mouth aspirator set (American Hospital Supply Corporation) with parafilm.

The appropriate discs were dissected in LRS (Lisa's Ringer's Solution, NaCl 7.5 g/l, KCl 0.35 g/l, 10 mM tricine, pH 7.2 and 100 mg/ml streptomycin made up in sterile Milli-Q distilled water, filter sterilized and kept frozen). Discs were fragmented in a thin film of LRS on a slide using a sharpened insect pin.

Mated Canton-S female hosts (24-48 hours post-eclosion) were anesthetized with ether and placed ventral side up on double sided sticky tape attached to a microscope slide. A disc fragment was aspirated with a small amount of LRS into the tip of the needle. Fine forceps were used to pinch the ventral abdominal cuticle and the fragment was injected in a posterior to anterior direction between the sternites. The host fly was then placed in a vial containing yeast-agar medium. The vial was laid on its side so that the host would not become stuck in the medium. The survival rate of injected hosts was generally greater than 50% but could vary between 0-100%. This apparently depended on the quality of the needle.

The fragments were cultured for up to seven days. Prior to fixing and staining, the implants were either dissected from the host abdomen in PBS (25 mM $NaPO_4$ buffer, pH 7.2, 140 mM NaCl), or the host abdomen was removed from the remainder of the carcass and dissected along the dorsal midline using fine scissors and placed in PBS.

II.8 Histochemical techniques

II.8.1 Staining for β-galactosidase activity

β-galactosidase activity was assayed in imaginal discs, disc fragments, embryos and ovaries with the same staining reagents and only the tissue preparation differed. All fixed, washed tissues were stained in 20 µl 8% X-gal (Bachem) (dissolved in dimethyl formamide and stored at -20°C) / ml staining solution (25 mM $NaPO_4$, pH 7.2, 140 mM NaCl, 3.1 mM $K_3[Fe(CN)_6]$, 3.1 mM $K_4[Fe(CN)_6]$). For ovaries and embryos, the staining solution was supplemented with 0.2% Triton X-100. In all cases the staining reaction was monitored to avoid overstaining. This could take from 15 minutes to overnight depending on the insertion strain.

Inverted larval heads, disc fragments, or implants inside host abdomens were stained essentially as in Simcox et al. (1989). Tissues were dissected in PBS and fixed in Glutaraldehyde-PBS, (30µl of 25% glutaraldehyde (Sigma)/ml PBS) for 15 minutes at room temperature. If many samples were being processed at once, the tissues were kept on ice prior to fixation for not more than 30 minutes. The tissues were washed three times in PBS prior to staining.

Embryo fixation was performed as described in Klämpt et al. (1991). Embryos were collected from egg laying plates, transferred into baskets and rinsed in distilled water. Reagents were distributed in 24-well tissue culture dishes (Falcon) and the embryos were transferred between solutions with the baskets. Embryos were dechorionated for 2-3 minutes with household bleach diluted 1:1 with distilled water. The embryos were rinsed twice with PBT (PBS + 0.2% Triton X-100) and fixed for 7 minutes at room temperature in heptane saturated with 2.5 % glutaraldehyde in PBS. They were then rinsed several times over the course of 1 hour in several changes of PBT, until the embryos no longer stuck together.

Ovaries were stained essentially as in Grossniklaus et al. (1989). Mated females were washed and dissected in PBS and the ovaries were tranferred to 24 well micro-titre dishes containing 30μl devitellinizing buffer (1 volume buffer B (10 mM KPO_4 (pH 6.8), 45 mM KCl, 150 mM NaCl, 2 mM $MgCl_2$): 4 of H_2O :1 of 37% formaldehyde) and 200 μl heptane. The plates were rocked gently for 10 minutes at room temperature, the solution was drawn off and replaced with 300 μl of 1% glutaraldehyde in PBS. The ovaries were washed in PBS and stained as above.

II.8.2 Immunostaining of imaginal discs and disc fragments

Imaginal discs and disc fragments were stained essentially as in Pattatucci and Kauffman (1991) with some modifications. Tissues were dissected in PBS and stored in 1.5 ml microcentrifuge tubes on ice for no more than 30 minutes. The PBS was drawn off and replaced with 280 μl PBS, 120 μl 10% paraformaldehyde in PBS (stored frozen at -20°C) and 500 μl heptane. The samples were shaken by hand for 60 seconds and the fix was replaced with 520 μl PBS, 240 μl 10% paraformaldehyde in PBS, and 40 μl fresh DMSO. After rocking gently at room temperature for 20 minutes, the discs were twice rinsed in methanol and washed for 30 minutes in 20 μl 30% H_2O_2 and 980 μl methanol. The samples were then washed four times for ten minutes each in PBNB (PBS+0.5% NP-40 (Sigma) + 0.1% BSA (Sigma)), and blocked for at least 30 minutes in PBNB + 5% skimmed milk powder. The primary antibody was added and the tissue was incubated overnight, gently rocking at 4°C. The concentration of primary antibody used was 1/20 000 for rabbit anti-β-galactosidase (Cappell), 1/100 for a mouse monoclonal anti-crumbs antibody (kindly provided by Dr. Elizabeth Knust) and 1/25 for a monoclonal anti-engrailed/invected (kindly provided by Dr. Pat O'Farrell). The antibody dilutions were determined empirically.

The primary antibody was washed off with 5 changes of PBNB over a period of 1.5 hours, and the tissue was reblocked for 30 minutes in PBNB + 5% skimmed milk powder. Biotinylated goat anti-rabbit (Vector) or biotinylated horse anti-mouse secondaries (Vector) were added to the blocking solution at a 1/250 dilution and and the samples were rocked for 90 minutes at 4°C. The secondary antibody was washed off with 4 changes of PBNB over a period of one hour. The Vectastain ABC reagent (Vector labs) was prepared according to the manufacturer's instructions, with 10 µl A reagent (streptavidin) and 10 µl B reagent (biotinylated peroxidase) per 500 µl of PBNB), and allowed to sit for 30 minutes prior to use. The final wash was removed and the tissue was incubated in the ABC reagent for sixty minutes, rocking gently at 4°C. The ABC reagent was removed and the samples washed twice in PBNB and three times in PBN for ten minutes each wash. The final wash with tissue sample was transferred to a 24-well tissue culture dish and the solution replaced with 450 µl of PBN, 50 µl of 5mg/ml DAB (diaminobenzidene (BRL)) in 0.1 M Tris-Cl , pH 7.2, and 5 µl 3% H_2O_2. The reaction was monitored with a dissecting microscope until acceptable staining had occurred (generally 5-10 minutes). At this point the reaction was stopped by rapidly washing with three changes of PBN.

When cultured disc fragments were being processed in host abdomens, inverted larval heads of the same genotype were included to monitor the extent of the staining reaction. Generally, overstaining the control larvae gave the best results for the implanted fragments.

If double staining was to be performed, after termination of the first DAB staining the tissues were incubated in 0.2 M Glycine, pH 2.5, for 5 minutes to strip off the primary antibody as in Kellerman et al. (1990). The tissues were then reblocked in PBNB+5% skimmed milk powder for two hours. The procedure above was then repeated from the point of addition of the second primary antibody, the only difference being that $NiCl_2$ or $CoCl_2$ was added to the final peroxidase staining reaction at a final concentration of 0.03%. Ni^{2+} and Co^{2+} result in a dark brown or dark blue DAB precipitate respectively. DAB alone gives a reddish brown precipitate.

II.8.3 Immunostaining of embryos

Immunostaining of embryos was performed as in Kellerman et al. (1990). Embryos were collected from egg-laying plates, transferred to an embryo basket, washed with PBT (PBS+0.2% Triton X-100), dechorionated with commercial bleach diluted 1:1 with distilled water and washed again before fixation. The embryos were fixed in equal volumes of 4% paraformaldehyde in PBS and heptane for twenty minutes on a shaker. As much of the aqueous layer as possible was removed and replaced with methanol. The embryos were shaken very vigorously by hand for 1-2 min. and then allowed to settle. The heptane phase was removed and replaced with more methanol and the embryos were shaken as before. The embryos were allowed to settle then they were washed three times in methanol. To the final methanol wash was added H_2O_2 to a concentration of 3% and the embryos were rocked at room temperature for 30 minutes to quench endogenous peroxidases. The embryos were then washed three times for five minutes in PBT and blocked in PBT + 5% skimmed milk powder for 2 hours. Rabbit anti-β-galactosidase (Cappell) was added to a final concentration of 1/4000 and the sample was gently rocked overnight at 4°C. The embryos were then washed 4 times over the course of 1.5 hours in PBT and reblocked in PBT + 5% skimmed milk powder for 30 minutes. The secondary antibody (biotinylated goat anti-rabbit, Vector) was added to a final dilution of 1/250 and rocked at 4°C for 90 minutes. The embryos were washed as after the primary antibody reaction and the DAB reactions were performed as for the imaginal discs except that the reactions were carried out in PBT rather than PBN.

II.8.4 Photomicroscopy and mounting of tissues

All embryo and disc tissues were stored and mounted in 1:1 PBS, glycerol. Antibody and X-gal-stained tissues could be stored in mounting medium for several weeks. With *in situ* hybridized tissues, the staining was found to fade after several days. Specimens were photographed with a Zeiss Axiophot photomicroscope (kindly provided by Dr. D. Pilgrim) using DIC optics. Kodak EKTAR 25 film was used and colour processing was done at HUB PHOTO, Hub Mall, University of Alberta campus.

II.9 DNA extraction

II.9.1 Genomic DNA extraction

Isolation of Drosophila genomic DNA was done using a protocol received from Dr John Locke, University of Alberta. Groups of 60 flies were placed in 200 µl of homogenization buffer (8 M urea, 10mM EDTA, 100 mM Tris, pH 8.0 and 2% SDS) in 1.5 ml screw cap tubes. The flies were homogenized using a thin 400 µl micro-centrifuge tube (Fisher Scientific) as a pestle until all the flies were fragmented into parts. The pestle was rinsed with a further 200 µl homogenization buffer, 50 µl of proteinase K (10 mg/ml) was added and the sample was incubated at 60°C for 2 hours. The tubes were removed and while they were still hot, 500 µl of chloroform was added and mixed by inversion for 30 seconds. The tubes were then centrifuged in a microcentrifuge for 2 minutes. The chloroform was removed and replaced with 500 µl phenol and the tube was mixed by inversion and centrifuged as before. The aqueous phase was transferred to a 1.5 ml snap-top microcentrifuge tube containing 500 µl of phenol, mixed by inversion and centrifuged as before. The second phenol was removed and replaced with 500 µl of chloroform. The tube was mixed by inversion and centrifuged as before then all of the chloroform was removed. Distilled water was used to bring the aqueous volume to 400 µl and the nucleic acids were precipitated with 1 ml of 95% ethanol. The tube contents were mixed by inversion, allowed to sit for 2 minutes at room temperature and centrifuged for 5 minutes to collect RNA and DNA. The pellet was washed with 70% ethanol, repelleted, and allowed to dry at room temperature. The pellet was then resuspended in 120 µl of TE (10 mM Tris, pH 8.0, 1mM EDTA) and stored at 4°C.

II.9.2 Plasmid Miniprep

Small scale preparations of plasmid DNA were carried out as in Sambrook et al. (1989). The optional STE and phenol-chloroform extractions were included and a 70% ethanol wash was inserted after the 95% ethanol precipitation.

II.9.3 Larger scale plasmid prep

A larger scale plasmid preparation was done using a modification of the mini-prep protocol from Sambrook et al (1989) (D. Pilgrim, pers. comm.). 10 ml of overnight bacterial culture was centrifuged for 8 minutes at 6000 rpm in 15 ml disposable plastic snap-cap tubes (Simport Plastics) in a Beckman preparative centrifuge. The supernatant was discarded and the pellet resuspended in 250 µl of GTE (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0). The resuspended pellet was transferred to a 1.5 ml microcentrifuge tube and put on ice for 20 minutes. About 0.5 ml of freshly prepared 0.2 M NaOH, 1% SDS was added, mixed by gentle inversion, and iced for 10 minutes. 250 µl of Solution 3 (60 ml 5 M potassium acetate + 11.5 ml glacial acetic acid + 28.5 ml distilled water/100ml) was added, mixed 10 times by gentle inversion and centrifuged for 10 minutes in a microcentrifuge in a 4°C cold-room. 800 µl of supernatant was transferred to a microcentrifuge tube and precipitated with 700 µl of isopropanol with thorough mixing. This was centrifuged at room temperature for 15 minutes, the supernatant discarded and the the pellet washed with 70 % ethanol. The ethanol was removed, the pellet was dried in a dessicator and resuspended in 200 µl of TE and 20 µl of 3 M sodium acetate, pH 5.3. The sample was extracted once with Phenol, once with 25:24:1 Phenol, Chloroform, iso-amyl alcohol and once with chloroform, transferring the supernatant to a fresh tube with each extraction. After the final extraction the aqueous phase was precipitated with 2 volumes of 95% Ethanol, and washed in 70% ethanol. The pellet was dissolved in 100 µl TE, and stored at 4°C.

II.10 DNA manipulation

II.10.1 Restriction Digests

Restriction enzymes were purchased from BRL, Pharmacia and Boeringher-Mannheim. Restriction digests were performed according to the manufacturers' suggestions with the buffers supplied. Heat-treated RNase A was added at a concentration of 25 µg / ml except in digests to produce templates for *in vitro* synthesis of RNA.

II.10.2 Gel Electrophoresis

Digested genomic or plasmid DNAs were separated in 0.5-1% agarose gels in either TAE buffer (40 mM Tris-acetate, 1 mM EDTA) or TBE buffer (89mM Tris-borate, 89mM boric acid, 2mM EDTA) including 0.5 µg/ml ethidium bromide. Samples were dissolved in a 5:1 mixture with 6X Dyes(0.25%

bromphenol blue, 0.25% xylene cyanol, 25% ficoll (type 400)) in H_2O. BRL 1 kb ladder was used as a size standard.

II.10.3 Gel Purification

Desired DNA fragments were excised from 0.5-1.0% TAE-agarose gels following electrophoresis. DNA was purified from the excised agarose plugs using the GENE-CLEAN kit (BIO 101) according to the manufacturer's protocol.

II.10.4 Plasmid rescue

Plasmid rescue was performed using a protocol communicated by Dr. W.R. Addison. Approximately 2.5 µg of G-45 genomic DNA was digested with *Xba* I. One half of the reaction was run on an agarose gel, and stained with ethidium bromide to ensure that digestion was complete. The other half was phenol-chloroform extracted, to inactivate the restriction enzyme and then added to a ligation reaction using BRL T4 DNA ligase buffer and 1U of T4 DNA ligase in a total volume of 500 µl. This was incubated at 15°C for 24 hours and heated for 20 minutes at 70°C to inactivate the ligase. The reaction-mix was concentrated to a 50 µl volume with a Spinvac and precipitated with 25 µl of 7.5 M ammonium acetate and 150 µl ethanol at -20°C for 1 hour. After centrifugation, the pellet was washed with 70% ethanol, dissolved in 100µl distilled H_2O and precipitated with 10 µl 3 M sodium acetate and 250 µl 95% ethanol at -20°C for 1 hour. After centrifugation, the pellet was washed 3 times with 70% ethanol and dissolved in 7 µl of half strength TE. Five µl of the sample was used to electroporate (2.3 kV for 5.3 msec.) 40 µl of competent DH5-α cells (provided by Laura Querengesser) with a BTX electroporator, used with the courtesy of Dr. W. Addison. Cells were allowed to recover in SOC medium for 1 hr and then were plated on LB plates supplemented with 50 µg/ml kanamycin. Positive colonies were grown in 1ml of LB + 50µg/ml kanamycin and plasmid DNAs were isolated.

II.10.5 Subcloning

Subcloning was performed using standard methods (Sambrook et al 1989). The 2.3 kB insert from pG-45-R was excised using *Hind* III and gel purified. Gel purified, *Hind* III digested Bluescript SK- was used as the vector. The vector and target DNAs were combined in a 1:4 ratio in a final volume of 14 µl with sterile water. Four µl of 5x T4 DNA ligase buffer (BRL) and 2 µl of T4 DNA ligase (1U/µl) was added. The reaction was left overnight at 15°C and was stopped by heating at 75°C for 10 minutes. One half of the reaction was used to transform DH5-α cells. The transformants were selected on McKonkey agar plates supplemented with 50 µg/ ml ampicillin.

II.10.6 Plasmid transformations

Transformations were carried out using DH5-α sub-cloning-competent cells generously supplied by Andrew Simmonds.

II.10.7 Labelling DNA with ^{32}P

Gel purified DNA fragments were labelled with α-^{32}P-dCTP (ICN) with an "oligo-labeling kit" (Pharmacia). Generally reactions were performed in 30 μl total volume with 30 ng heat denatured DNA and 30 μCi of labelled nucleotide as per the instructions of the manufacturer. The unincorporated label was separated from the labelled DNA using standard Spin-Column chromatography (Sambrook et al., 1989).

II.10.8 Labelling DNA with Digoxigenin-dUTP

Labelling of gel purfied DNA fragments with digoxigenin-dUTP was carried out using the "DIG DNA Labelling and Detection Kit" (Boehringer Mannheim) following the manufacturer's instructions for probe preparation and separation of unincorportated nucleotides. Generally, 100ng of DNA was used per reaction and the reaction was allowed to proceed at 37°C overnight.

II.10.9 Synthesizing Digoxigenin labelled RNA in vitro

Digoxigenin labelled RNA was synthesized according to a protocol suggested by Dr. Ron Blackman. One μg of linearized gel purified template DNA was used in a total volume of 5 μl. The total reaction volume used was 10 μl and included the 5 μl of template, 2 μl of 5X transcription buffer (Promega), 0.5 μl of 100mM dithiothreitol, 0.5μl RNAse inhibitor (100 U/μl, Promega), 1μl of 10X Digoxigenin-RNA labelling mixture (Boehringer Mannheim) and 1μl of T3 or T7 RNA polymerase (Promega). The reaction was allowed to proceed for 2 hours at 37°C. 1μl of the reaction was run on a TBE gel. Normally a distinct product is visible under these conditions. To the remainder of the reaction was added 16 μl of sterile water, and 25 μl of 2X carbonate buffer (120 mM Na_2CO_3, 80mM $NaHCO_3$, pH 10.2, stored as frozen aliquots which are discarded after one use). The mixture was incubated at 65°C for 20 minutes to reduce the size of the *in vitro* synthesized transcript. The reaction was stopped with 50 μl 0.2M sodium acetate, pH 6.0 and precipitated at -20°C with the addition of 10 μl 4M LiCl, 5μl 20 mg/ml phenol/chloroform-extracted Torula RNA (Sigma) and 300 μl 95% ethanol. The precipitate was recovered by centrifugation in a microcentrifuge for 20 minutes at 4°C. The pellet was washed in 70% ethanol, resuspended in 100 μl sterile H_2O, and stored frozen at -20°C.

II.11 Hybridizations

II.11.1 *In situ* hybridization to polytene chromosomes

Salivary glands from late third instar of the appropriate genotype grown at 20°C were dissected in 45% glacial acetic acid and placed in a small drop of 45% acetic acid on a siliconized cover slip. The cover slip was picked up with a subbed slide (coated in 0.5% gelatin) and squashed according to the method of Pardue (1986), except that the final squash was performed with a the help of a C-clamp and a Staedtler eraser. The slides were fixed in 3:1 95% ethanol, glacial acetic acid for 60 seconds and dehydrated twice for 10 minutes each in 95% ethanol. The squashes were assessed for flatness after being allowed to dry at room temperature for 30-45 minutes. Slides could be stored at this stage for several weeks at 4°C. Slides were processed according to a previously published method (starting at step 1 F of Engels et al., 1986) with the following changes. The heat treatment was done at 58°C rather than at 65°C, and the 2X SSC washes following the NaOH treatment were omitted.

Hybridization was performed using a protocol from Dr J. Locke. The probe was prepared by first resuspending a complete Digoxigenin DNA labelling reaction in 5 µl sterile H_2O. To this was added 21 µl of 20X SSC (3M NaCl, O.3M Na citrate, pH 7.0), 2.3 µl 25mg/ml yeast tRNA, 13.7 µl 10mg/ml sonicated salmon sperm DNA, 13 µl 50% dextran sulphate, and 45 µl de-ionized formamide (stored at -20°C) for a total volume of 100 µl. The probe was denatured by heating at 80°C for 10 minutes and then quick-cooled on ice for 3 minutes. Twelve to fifteen microlitres of the denatured probe was pipetted onto the squash and was covered with a 22 mm square coverslip and placed in a slide box with Kleenex soaked in water stuffed in the bottom (a "moist chamber") and hybridized at 37°C, overnight.

Posthybridization washes were performed as in Engels et al. (1986) with the following changes. The PBS washes were omitted and the Boehringer Mannheim blocking agent (0.5%) was susbstituted for BSA. Instead of the streptavidin and biotinylated alkaline phosphatase steps, a single step using alkaline phosphatase-conjugated rabbit anti-digoxigenin Fab' fragments at a dilution of 1/1000 in buffer #1 was used. The staining reaction was similar except that the Boehringer Mannheim nitro-blue tetrazolium and X-phosphate, 4.5 µl and 3.5 µl/ per ml of buffer #3 respectively, were used. Staining was generally found to be satisfactory after 1-2 hours of the staining reaction. When longer staining was required to see the signal, the background staining became quite high. The staining reaction was stopped by washing the slides in TE. The slides were stored at 4°C.

II.11.2 *In situ* hybridization to embryo and imaginal disc RNA

Embryos and imaginal disc RNA were hybridized in situ using a very similar protocol (based on Tautz and Pfeiffle, 1989) and the main differences involved the initial fixing procedure. All aqueous solutions, excluding 10%

paraformaldehyde in PBS, were DEPC (Sigma) treated (Sambrook et al., 1989) prior to addition of Tween-20 (Fisher Scientific).

Embryos were collected from egg-laying plates, transferred to an embryo basket and washed with PBTw (PBS + 0.1% Tween-20) and dechorionated with commercial bleach diluted 1:1 with distilled water and washed again with PBTw before fixation. The embryos were fixed in equal volumes of 4% paraformaldehyde in PBS (made from a 10% paraformaldehyde in PBS stock solution diluted with PBS) and heptane for twenty minutes on a shaker. As much of the aqueous layer as possible was removed and replaced with methanol. The embryos were shaken very vigorously by hand for 1-2 minutes and then allowed to settle. The heptane phase was removed and replaced with more methanol and the embryos were shaken as before. The embryos were allowed to settle then they were washed three times in methanol. The embryos were then rehydrated though a series of three five minute methanol/4% paraformaldehyde in PBS washes (700 μl:300μl, 500 μl: 500μl, 300 μl:700μl), and then for 20 minutes in 4% paraformaldehyde in PBS. At this point, the embryos were washed in PBTw for 5 minutes and dehydrated through an ethanol series (30%, 50%, and 70%) and stored at -20°C until ready to use.

Imaginal discs and disc fragments were stained essentially as in Pattatucci and Kauffman (1991) with some modifications. Tissues were dissected in PBS and stored in 1.5 ml microcentrifuge tubes on ice for less than 30 minutes. The PBS was drawn off and replaced with 280 μl PBS, 120 μl 10% paraformaldehyde in PBS and 500 μl heptane. The samples were shaken by hand for 60 seconds and the fix was replaced with 520 μl PBS, 240 μl 10% paraformaldehyde in PBS, and 40 μl fresh DMSO. This was rocked gently at room temperature for 20 minutes. The discs were washed three times for five minutes each and dehydrated through an ethanol series (30%, 50%, and 70%) and stored at -20°C until ready for use.

Embryos and discs were rehydrated though an ethanol series (70%, 50%, and 30%) and washed three times at room temperature in PBTw. To the last wash was added proteinase K to a final concentration of 50 μg/ml. Imaginal discs were shaken by hand for 45 seconds; embryos were rocked on a platform shaker for 8 minutes. The tissues were washed two times for five minutes each in PBTw + 2mg/ml glycine, washed three times for five minutes each in PBTw and then refixed in 4% paraformaldhyde, 0.2% glutaraldehyde in PBS for 20 minutes at room temperature. After fixing, the tissues were washed five times for five minutes each in PBTw.

The tissues were washed for 10 minutes in 1:1 PBTw, hybridization solution (50% deionized formamide, 5X SSC, 100 μg/ml sonicated salmon sperm DNA, 200 μg/ml torula RNA, 0.1% Tween-20) without probe and then for 10 minutes in hybridization solution without probe. The tissues were then prehybridized for at least one hour at 58°C rotating in a hybridization oven. DIG-labelled RNA (see above) dissolved in sterile water was added to hybridization solution (generally 1 to 5 µl/100µl hybridization solution was found to be optimal) and denatured by heating at 80°C for 5 minutes and cooled quickly on ice. Tissues were hybridized for 24-36 hours at 58°C.

Posthybridization washes were as follows: hybridization solution, two times rapidly at room temperature, followed by two times 20 minutes at 58°C. These were followed by a series of hybridization solution, PBTw washes (4:1,3:2,2:3,1:4) at 58°C for 20 minutes each. This series was followed by two washes in PBTw at 58°C for 20 minutes each.

The tissues were blocked in PBTw + 0.1% BSA (bovine serum albumin, crystalline grade, Sigma), two times, for twenty minutes each, and immersed in a 1/1500 dilution of alkaline phosphatase-conjugated rabbit anti-DIG Fab' fragments in PBTw + 0.1%BSA. Prior to use, the conjugate was adsorbed to fixed larval heads. The binding was allowed to proceed for 90 minutes and the conjugate was washed off with three washes (20 minutes) in PBTw+0.1%BSA. The tissues were then washed three times in alkaline phosphatase buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-Cl, pH 9.5, 10 mM Levamisol (Sigma), 0.1% Tween-20). To the last wash was added, 4.5 µl Nitro blue tetrazolium (Boehringer Mannheim) and 3.5 µl X-phosphate (Boehringer Mannheim) per ml. The reaction was done in the dark and was monitored periodically under a dissecting microscope. When the reaction had proceeded sufficiently, it was stopped by washing three times rapidly in PBTw.

II.11.3 Southern Hybridization

DNAs were digested and electrophoresed though 0.7% agarose-TBE gels. Southern transfers by capillary blot were set up with the GeneScreenPlus membrane (Dupont) and hybridizations were performed as specified by the manufacturer as described in the manual supplied with the membrane. Generally, 10^6 Cerenkov counts were added per ml of hybridization solution and the hybridizations were performed in tubes in a Tyler hybridization oven.

III. IDENTIFICATION OF ENHANCER-TRAPS EXPRESSED IN REGENERATING IMAGINAL DISCS.

This chapter describes an enhancer-trap screen for genes expressed during imaginal disc regeneration. Enhancer-traps identify patterns of gene regulation (O'Kane and Gehring, 1987). A collection of random autosomal enhancer-trap insertions was first tested for altered expression under conditions which induce cell death and consequent imaginal disc regeneration, in a temperature sensitive cell-lethal mutant, or following γ-irradiation. In a more direct test, a sample of the positives from these screens was examined for expression in regenerating or duplicating cultured disc fragments. Finally, their *lacZ* expression patterns during embryogenesis and oogenesis were determined in order to assess the possible functions of the genes identified by the insertions.

III.1 Identification of enhancer-trap insertions expressed in imaginal discs following the induction of cell death

III.1.1 Analysis of enhancer-trap *lacZ* expression in the *suppressor-of-forked* cell-lethal background

Over eight hundred independent enhancer-trap insertions were generated using the single-hop method of Cooley et al., (1988) which is described in the methods section. I used the PZ element (Jacobs et al., 1989) (Figure 3) which carries an *E. coli lacZ* reporter gene fused in frame to the P-transposase promoter, which may be *cis*-activated when the transposon inserts near an enhancer of an active gene. Thirteen insertions of another enhancer-trap containing a similar promotor fusion (O'Kane and Gehring, 1987) were kindly supplied by Dr. J.Merriam , UCLA.

Each insertion line was crossed to *su(f)*^{12} (previously called *l(1) ts 726)* a temperature-sensitive, cell-autonomous lethal allele of *suppressor-of-forked.* A 48 h heat pulse at 29°C, the restrictive temperature, during larval development causes imaginal disc cell death in mutant larvae and efficiently produces disc fragments that regenerate or duplicate *in situ* (Russell 1974; Clark and Russell, 1977; Girton and Kumor, 1985). This makes it is possible to initiate regeneration in a large sample of insertion lines without resorting to time-consuming surgical fragmentation and *in vivo* culture. Males from each insertion line were crossed to females homozygous for the *y v f su(f)*^{12} chromosome and the cross was maintained at 22°C. When wandering stage third instar larvae appeared, the adults were transferred to fresh food vials, and the larvae were shifted to 29°C for 48h, followed by a 24 h recovery at 22°C. Imaginal discs from hemizygous cell-lethal male larvae and heterozygous female controls were stained for β–galactosidase activity with X-gal and examined with a stereomicroscope. Insertion lines were saved when the staining pattern of discs from cell-lethal males and control females differed. The primary screen and examples of β–galactosidase staining from a positive insertion line are shown in Figure 5.

Figure 5. Crossing scheme used to generate cell-lethal and control larvae carrying PZ insertions. The cross and subsequent treatments were performed for each insertion line. A: Eye-antenna disc from control female. Localized acridine orange staining in antennal disc indicates a low level of normal programmed cell-death (Spreij, 1971). B: Eye-antenna disc from heat-pulsed *su(f)*^{12} male with greatly increased punctate acridine orange staining indicating cell death induced by the cell-lethal mutation. C: Eye-antenna disc from heat-pulsed *su(f)*^{12}*/+; D-42/+* females with no detectable β-galactosidase activity. D: Eye-antenna disc from heat-pulsed *su(f)*^{12}*; D-42/+* male with substantial β–galactosidase activity distributed in a pattern similar to the induced cell-death (compare B and D). This figure is reproduced from Brook et al. (1993) with the permission of The Company of Biologists, Ltd.

1) Cross at 22°C

$$\text{y v f su(f)} \;,\; \frac{+}{+} \quad \times \quad \frac{+}{\urcorner} \;,\; \frac{\text{PZ[lacZ, ry}^{+}\text{]}}{\text{PZ[lacZ, ry}^{+}\text{]}}$$

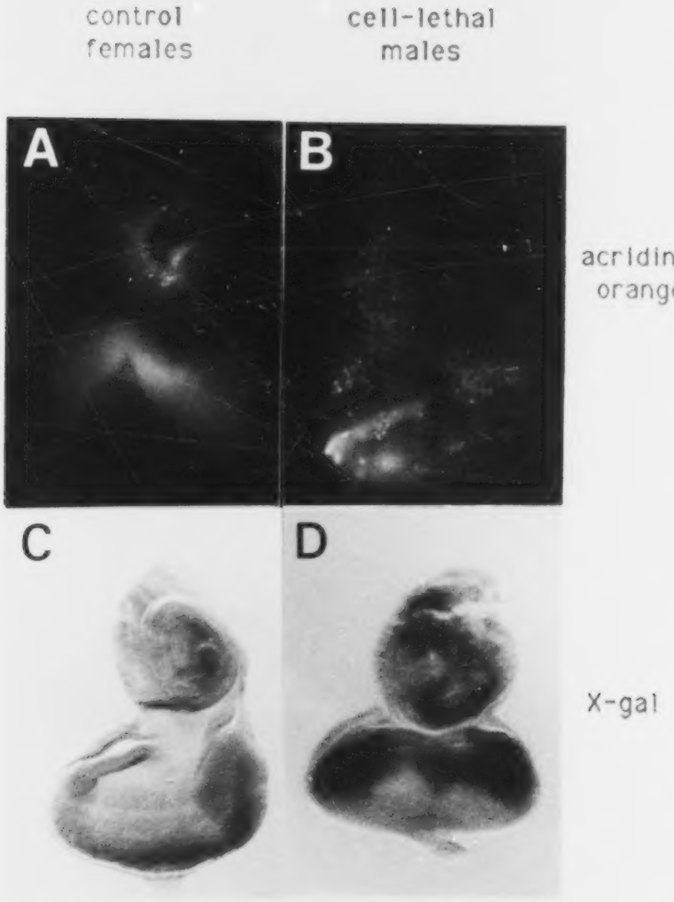
$$\downarrow$$

2) Shift culture to 29°C for 48 hours when third instar larvae appear

3) Shift culture to 22°C for 24 hours

4) Separate larvae by sex and recover imaginal discs

$$\frac{\text{y v f su(f)}}{+} \;,\; \frac{\text{PZ[lacZ, ry}^{+}\text{]}}{+} \qquad\qquad \frac{\text{y v f su(f)}}{\urcorner} \;,\; \frac{\text{PZ[lacZ, ry}^{+}\text{]}}{+}$$

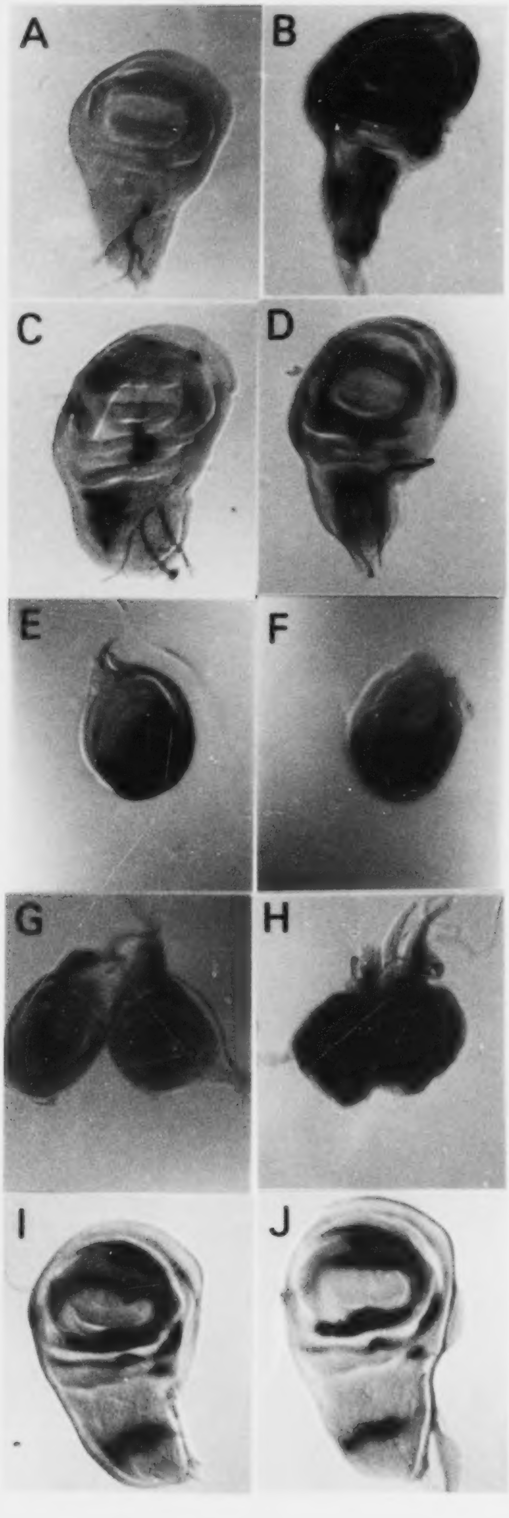

control
females
cell-lethal
males
A
B
acridine
orange
C
D
X-gal

A total of 826 autosomal insertion lines was screened for altered expression in the cell-lethal mutant background. The *lacZ* expression of 38% (312/826) of the lines tested was altered in some way. The expression of *lacZ* was unchanged in the remainder. The affected lines included a group of insertions expressed only in cell-lethal and not in control discs (82 lines), insertions expressed in an altered pattern (229 lines), and one expressed in controls but not mutant discs. Some examples are shown in Figure 6. The large class of lines in which a control expression pattern was altered cannot be explained merely by physical distortion of the disc epithelium due to cell death, because equally complex control expression-patterns were unchanged after treatment in most of the lines (e. g. Figure 6 I, J). Furthermore, the amount of staining induced in treated discs varied between discs from different lines and between discs from the same line. The induced staining can be seen to vary substantially in the five examples of disc staining (shown in Figure 6) from perhaps only a few cells expressing *lacZ* ectopically (Fig 6 F), to a majority of cells inducing *lacZ* expression following treatment (Figure 6 B). This excludes the possibility that there is a uniform response of the transposon to this genetic background.

Many insertions shared very similar patterns of *lacZ* expression in the cell lethal background. This was especially apparent in the eye-antennal disc. Nearly half of the insertions (153/312) had *lacZ* staining in front of the approximate position of the morphogenetic furrow (see Figure 5). This is the location of the highest frequency of imaginal disc cell death and pattern defects induced by heat pulses in *su(f)*^{12} (Russell, 1974; Clark and Russell, 1977). Thus, the expression in many of the positive insertion lines correlates well with the sites of cell death and presumably, wound healing and regeneration.

The question arose as to whether the response of the enhancer-traps to the cell lethal mutant is part of the cause or the effect of induced cell death. Mutants of *suppressor-of-forked* modify expression of gypsy-element insertion alleles at several loci. It has been reported that *su(f)*$^{+}$ negatively regulate gypsy transcript levels (Mazo et al., 1989). Sequence analysis of *suppressor-of-forked* reveals that it is homologous to *RNA14*, which encodes a yeast protein involved in mRNA stability (Mitchelson et al., 1993). As it is an essential gene, *su(f)* may similarly influence expression of certain normal genes. To see whether any of the responding insertions might be at loci regulated fortuitously by *su(f)*, rather than in genes participating in regeneration, the positives were retested for expression during the heat-treatment before regeneration is initiated. Expression was altered in 112 of 312 lines after 24 hours at 29°C. Since 24 hour temperature shifts do not cause any pattern defects and cell-death in this system only becomes evident later during the shift to the restrictive temperature (Clark, 1976), insertions affected as early as 24 hours must be responding directly to the *su(f)*^{12} lesion and not as genes participating in regeneration. This left 200 lines in which *lacZ* expression is affected only after regeneration has been initiated.

Figure 6. *lacZ* expression patterns in control (left-hand column) and cell-lethal (right-hand column) discs from five PZ insertion lines. A: Control and B: cell-lethal D-42 wing discs showing strong induction of beta-galactosidase in a complex pattern throughout the disc. C: Control B-93 wing disc with strong localized notum expression and in addition weak, punctate expression forming a cross in the wing pouch. D: cell-lethal B-93 wing disc with weak β-galactosidase activity in the notum and encircling wing forming region. The cross is no longer visible. E: control H-15 metathoracic leg disc with expression apparently restricted to the ventral compartment. F: cell-lethal H-15 metathoracic leg with ectopic stain in dorsal tarsus-forming region indicated by the arrow. G: control E-32 prothoracic leg discs staining at or near the anterior-posterior comparment boundary. H: cell-lethal E-32 prothoracic leg discs with staining greatly expanded along the A/P boundary and additional weak staining remote from the boundary in both anterior and posterior compartments. I and J, control and cell lethal H-09 wing discs with no detectable differences in staining. All single discs are oriented with the anterior compartment to the left. Wing discs are ventral side up; leg discs are ventral side down. This figure is reproduced from Brook et al. (1993) with the permission of The Company of Biologists, Ltd.


A
B
C
D
E
F
G
H
I
J

III.1.2 Analysis of *lacZ* expression following γ-irradiation

To test whether the insertions respond to an alternate source of cell-death, as expected if they are indeed in regeneration genes, discs from each of the positive lines were assayed 8 hours after exposure of third instar larvae to a 2500 rad dose of γ-radiation, and compared to discs from untreated larvae at the same developmental stage. It has been estimated that this dose kills 30% of cells in imaginal discs within 4 hours after treatment. By 8 hours, surviving cells re-enter the cell-cycle and begin to replace lost tissue (James and Bryant, 1981). The *lacZ* expression of 63 of 312 lines was judged to be altered by this treatment.

The two secondary screens defined four classes of insertion among those initially selected as positive (Figure 7). The 44 Class I insertions responded at the appropriate time to both kinds of cell-death, and thus, seemed the most likely to be involved in regeneration. The 156 Class II responded at the appropriate time in the *su(f)* background so they were also reasonable candidates, but they did not respond to γ-irradiation induced cell-death. Class III and Class IV were judged to be the least promising as they responded prior to the onset of cell-death in the *su(f)* background.

III.2 Characterization of enhancer-trap *lacZ* expression patterns

III.2.1 Expression patterns in control imaginal discs

Most of the positives also expressed *lacZ* in control discs. Table 2 summarizes the staining patterns of wing, haltere, leg and eye-antenna discs from control larvae. The incidence of different control expression-patterns varies strikingly among the five classes of insertion defined by the screens.

The most frequent control expression-pattern among Class V (nonresponding) lines is uniform, accounting for 47% of the sample scored. This pattern is much less frequent among the positive lines, especially in Class I, where it accounts for only 5% of the total. The next most common pattern in control discs is non-expression (Figure 6 A). This category is again under-represented in the Class I (9% of Class I but 27% of all other lines). The remaining lines are expressed in spatially non-uniform patterns in control discs (Figure 6 C, E, G and I). Interestingly, this category is much more common among lines that respond to the primary screen, and the over-representation is most marked in Class I (86% of Class I, 67% of Class II, 53% of III, 59% of Class IV, and 25% of Class V). These results suggest that pre-existing patterned gene expression in the imaginal discs may be important for pattern respecification in regeneration.

Figure 7: Definition of the five classes of insertion lines according to the primary and secondary screens. The numbers in parethese indicate the distribution of the 826 insertions according to each test criterion. This figure is reproduced from Brook et al. (1993) with the permission of The Company of Biologists, Ltd.

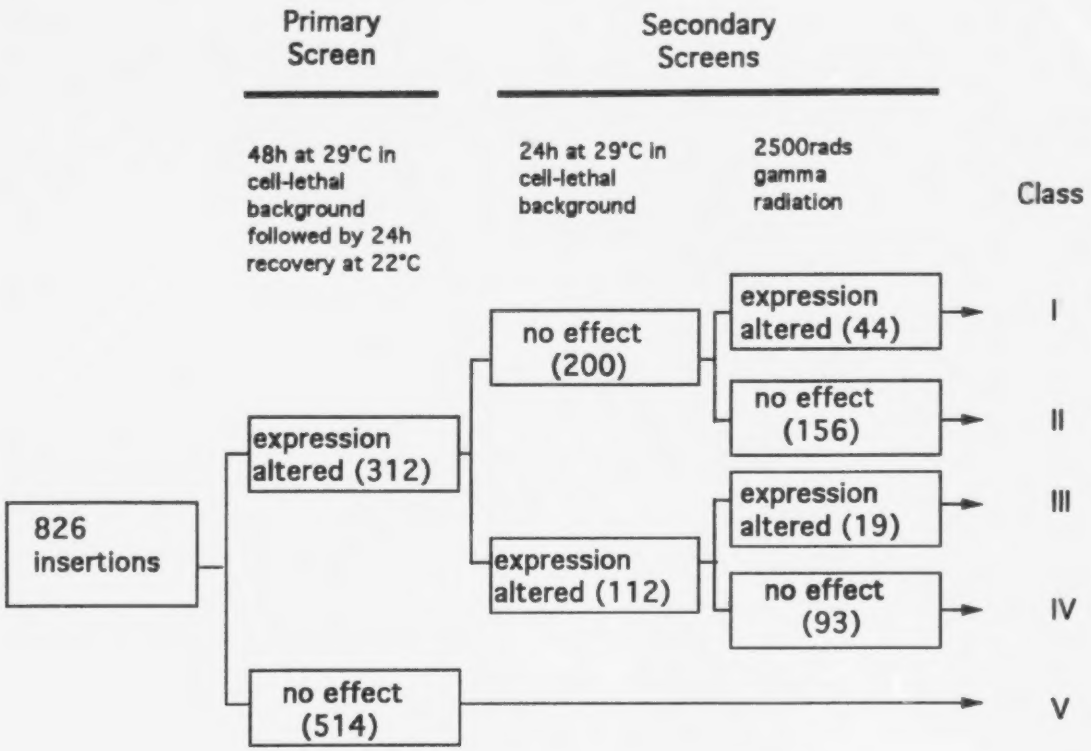

Primary
Screen
Secondary
Screens
48h at 29°C in cell-lethal background followed by 24h recovery at 22°C
24h at 29°C in cell-lethal background
2500rads gamma radiation
Class
826 insertions
expression altered (312)
no effect (514)
no effect (200)
expression altered (112)
expression altered (44)
no effect (156)
expression altered (19)
no effect (93)
I
II
III
IV
V

Table 2: Control staining patterns in imaginal discs from insertion lines of each class defined by the screens

lacZ expression pattern[a]	Class of insertion I (n=44)	II (n=156)	III (n=19)	IV (n=93)	V (n=375[b])	total (n=687)
no expression	4	45	7	26	106	188
uniform expression	2	6	2	12	177	199
following morphogenetic furrow in eye disc[c] and distal portions of leg and antenna discs[d]	17	53	5	29	27	131
distal portions of leg and antenna discs[d]	8	29	2	20	10	69
proneural (wing margin or discrete spots in discs)	2	5	0	0	3	10
following morphogenetic furrow in eye disc[c]	1	3	0	0	6	10
compartment boundary	6	9	0	0	1	16
compartmental	1	3	0	0	5	9
other	3	3	3	6	40	55

[a] Strains are categorized by the most prominent staining pattern exhibited.

[b] Data from a sample of class V lines (375 of 514) are shown.

[c] Expression in all or most of eye disc following the morphogenetic furrow and in a few cases slightly in front of the furrow as well.

[d] Similar restricted expression in corresponding folds of leg and antennal discs.

The particular expression patterns found preferentially in the target Class were as follows (see Table 2). The most common was a pattern that included corresponding parts of distal antenna and leg. Interestingly, most of these lines also expressed *lacZ* at or posterior to the morphogenetic furrow in the eye-disc (e. g. Figure 14 C). The incidence of this expression-pattern was enhanced about five-fold among insertions that responded to the primary screen. In another group of lines control expression was in discrete spots along the putative wing margin and/or notum in patterns similar to proneural and neurogenic genes involved in development of the imaginal sensory nervous system (e.g. Figure 6 C). This pattern was about two-fold more common among the selected lines. Finally, two groups of lines were expressed in control discs in patterns correlated with the compartmental subdivisions. Compartmental expression patterns (e. g. Figure 6 E) were about equally frequent in responding and non-responding classes, but 15 of the 16 lines which showed expression along a compartment boundary (e. g. Figure 6 G) were found in Class II. For three of the lines (AD-55, E-32 and H-39) the approximate A/P boundary site of expression was confirmed by their co-incident expression in double-insertion combinations with each other and with an *engrailed* enhancer-trap insertion, ryXho25 (Hama et al., 1990). The high proportion of the compartment boundary class in the positive lines was also of interest because of the timepoint chosen to assay β-galactosidase activity in the treated discs in the primary screen. Girton and Russell (1980) found that 24 h after a 48 h larval heat pulse somatic clones induced in the regeneration blastema reacquired a restriction to either the anterior or posterior compartment in *su(f)* induced duplicate legs.

III.2.2 Embryonic expression of Class I and Class II insertion lines

The embryonic expression of the Class I and Class II insertions was determined by X-gal staining. Particular attention was paid to embryonic stages prior to germ-band retraction as most of the events controlling the establishment of segments and imaginal disc primordia have occurred by this stage (Cohen 1993). The majority of insertions (118/185) had some expression prior to germ band retraction and the occurrence of complex patterned expression was very frequent (Table 3).This is consistent with reported samplings of enhancer-trap expression patterns (Bier et al 1989; Bellen et al, 1989). Many of the insertions have expression patterns suggestive of roles in gastrulation, neurogenesis, or segmentation.

The embryonic expression patterns allow for some speculation regarding the function of the genes detected by the enhancer-traps. This is especially useful in cases where the enhancer-trap has no expression in control imaginal discs. Of the fifty Class I and Class II insertions which did not stain in control imaginal discs there were examples of expression in all tissue types and in various patterns. While this analysis is not particularilt informative, it does suggest that genes from a variety of embryonic processes may be recruited into imaginal disc development during regeneration.

Table 3: Stage of first detectable *lacZ* expression and embryonic expression patterns in class I and II insertions

stage of first expression		class I	class II	Total
blastoderm		5	13	18
gastrulation		10	31	41
germ band extension		17	53	70
after germ band retraction		1	9	10
no embryonic expression		7	39	46
not determined		4	11	15
Total		44	156	200
Early expression patterns (before the completion of germ band extension)				
anterior/posterior (embryonic termini)		24	66	90
	ant	24	66	90
	post	3	3	6
	ant + post	3	3	6
cephalic furrow		7	19	26
germ band expression		16	47	63
	homogeneous	4	2	6
	pair-rule expression	8	32	40
	segmental	0	2	2
	inner gerband	0	7	7
Later expression				
Ectoderm		17	39	56
	trunk (T1-A8)	1	0	1
	ventral, segmental	2	5	7
	lateral, segmental	5	14	19
	dorsal, segmental	2	22	24
	full segmental	6	10	16
	salivary gland	0	1	1
	trachea	1	1	2
	pharynx	0	2	2
	spiracles	0	4	4
Mesectoderm		1	5	6
Mesoderm		6	13	19
	somatic	4	12	16
	visceral	2	3	5
	heart	0	1	1
Gut		5	18	23
	anterior midgut	2	9	11
	posterior midgut	4	9	13
	foregut	0	4	4
	midgut	0	7	7
	hindgut	1	6	7
	malphigean tubules	1	1	2
Neural		5	22	27
	brain	0	11	11
	cns	1	14	15
	pns	2	10	12

III.2.3 Expression of enhancer-traps in adult ovaries

More than half of the twenty-three insertions expressed in cultured disc fragments (see below) also showed *lacZ* expression in the adult ovary (see Table 7 at the end of this chapter). Nine lines stained in somatic follicle cells or germarium, and three in nurse cells. Several lines (C-76, E-60, C-07, H-87, AD-55) stained in a subset of follicle cells, e.g. in polar follicle cells as shown for C-07 in Figure 14 C. As the figure shows, this line is also expressed posterior to the furrow in the eye disc, but only after injury, at the wound heal, in wing discs (compare figures 14 A and 14 B).

III.3 Expression of enhancer-trap insertions in cultured disc fragments

Analysis of the enhancer-traps in cultured disc fragments is the most direct test to determine if they are genuinely expressed during regeneration or duplication. With this test, it is possible to correlate the temporal and spatial pattern of enhancer-trap expression with formation of a regeneration blastema. Although too time consuming for initial screening, disc transplantation and culture was used to evaluate the success of the screens. A sample of positives including insertions from classes I, II, III, and IV was tested in this manner.

III.3.1 Enhancer-trap insertions not expressed in control imaginal discs

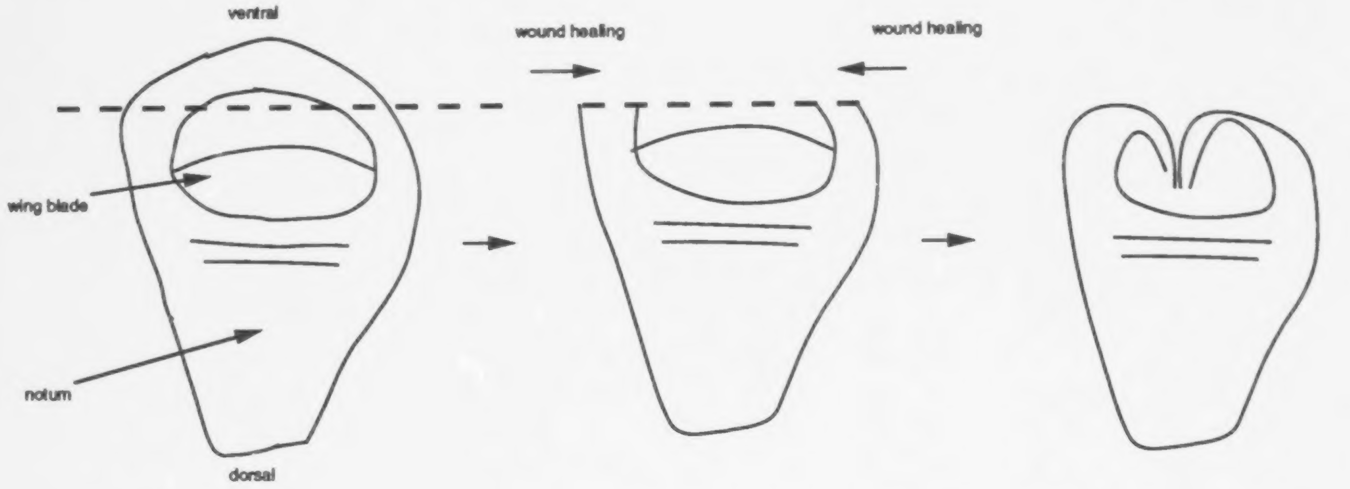
Insertions with no expression in untreated imaginal discs were a convenient choice for this test. Estimates of the size of the regeneration blastema vary from as few as ten cells, estimated by clonal analysis in *su(f)* induced leg duplicates (Girton and Russell, 1980) to hundreds of cells, estimated by labelling of S-phase nuclei in cultured surgical wing disc fragments (O'Brochta and Bryant, 1987). In either case, it was likely that expression would be detectable because of the absence of pre-existing expression in the disc. These insertions are also of considerable interest as they may represent genes not required for normal disc development that are activated specifically during disc regeneration.

Wing imaginal discs were dissected from late third instar larvae from insertions lines with no control staining patterns. The dissected discs were bisected using the "06" cut of Bryant (1975) (see Figure 8) and the larger, regenerating fragment was injected into adult female hosts, recovered following either one or two days culture, and assayed for *lacZ* expression with X-gal or with an immunohistochemical stain for an antibody to the β-galactosidase protein (see methods).

Stained fragments were first scored under a dissecting microscope and then under differential interference contrast (DIC) optics. No staining was observed in the epithelium of cultured, stained control Canton-S wing-disc fragments (n=16). Staining was occasionally seen in material adhering to the surface of the fragments. This material may have come from the host but could be easily distinguished from the epithelial expression observed in fragments

Figure 8: Diagram indicating location of cut to generate "06" fragment (Bryant (1975). The fragment is bissected through the wing pouch, and the larger regenerating fragment is injected.

06 CUT


ventral
wing blade
notum
dorsal
wound healing
wound healing

from the enhancer-trap lines with DIC optics, or with very careful examination of the fragment under a dissecting microscope.

In cultured fragments from many of the enhancer-trap lines, *lacZ* staining was clearly localized to the disc epithelium. Examples of this expression can be seen in Figure 9. The *lacZ* expression in a fragment from the G-45 insertion (Figure 9 A, C) is restricted to a small number of cells adjacent to an apparent site of wound healing. This is interpreted as the site of wound healing based on its location relative to other morphologically distinctive features in the fragment such as the notum (Figure 9 A (N)), and the prominent discontinuity in the epithelial cell layer (Figure 9 A, arrow), consistent with the folding together of the cut edges of the fragment (Reinhardt et al., 1977). The *lacZ* expression in this fragment is also restricted to one side of the wound (Figure 9 C, arrow). In some lines (C-76, E-34, E-37, and E-60), staining was also observed in cells distant from the site of the wound. Figure 9 B, D shows a fragment from the insertion E-37. Staining is clearly in the epithelium near the wound site (marked with arrow) and also quite distant from the wound in the hinge and notum regions of the fragment (marked with N). This may indicate that secondary lesions were induced in the fragment during the process of injection. Alternatively, some of the insertions may be expressed in cells not directly involved in wound healing or blastema formation.

Sixty-nine of eighty-two insertion lines with no staining in control imaginal discs were tested as described above. Fifteen lines tested positive and all but one of these was from classes I or II (see Table 4). The tests were scored as positive when at least one in a sample of recovered fragments for an insertion line demonstrated staining in the disc or peripodial epithelium. It is not necessarily expected that all of the fragments in a sample should stain for *lacZ* as the extent of regeneration and wound healing can vary greatly depending on how well the fragment heals following injection. Indeed, many implanted discs completely fail to regenerate. It is likely that some of the 54 lines which tested negative did so because of inadequate sampling (see Table 5). Only 12% (6/47) of lines where 1-5 discs were sampled scored positive (mean #= 3.5 for positives, 3.2 for the entire sample), while positives were almost four times as frequent (45%, 9/20) when 6 or more discs were sampled (mean#= 8.7 for positives, 7.7 for the entire sample) (see Table 5). However, the preponderance of Class I and Class II insertions among the positives can not be explained by this sampling bias. The proportions of lines for classes II, III, and IV in the lower sample range (1-5 disc fragments) are quite similar (71% mean #= 3.0, 67% mean #= 4.0 and 79%, mean #= 3.0, respectively). The proportion of Class I lines sampled at the lower range is smaller (50%, mean #=3.5), but both the negative lines are in the higher sample range.

As an additional control, larvae from all of the insertions found to be expressed in cultured disc fragments were subjected to a two hour, 37°C heat shock, at the suggestion of Dr. Robert Tanguay, Université de Laval. It is of interest that the only insertion which was induced by the treatment was the line C-39, the exceptional line from Class III. This suggests that this line may respond to several sources of cellular stress.

Figure 9: Ectopic *lacZ* expression in cultured G-45 and E-37 "06" wing disc fragments cultured in vivo. A: G-45 wing disc fragments cultured in vivo for 48 h and immunostained for *lacZ*. Ectopic expression is restricted to the region of the healed wound (arrow) and is not seen in regions distant from the wound (i.e. in the notum, N). B: E-37 wing disc cultured in vivo for 48 h and immunostained for *lacZ*. Ectopic expression is seen at the wound site (arrow) and in regions distant from the wound (small arrow). C: Higher magnification of the healed wound in the G-45 fragment in panel A. Ectopic expression is clearly restricted to one side of the heal. D: Higher magnification of the site of wound healing of the E-37 fragment from panel B.

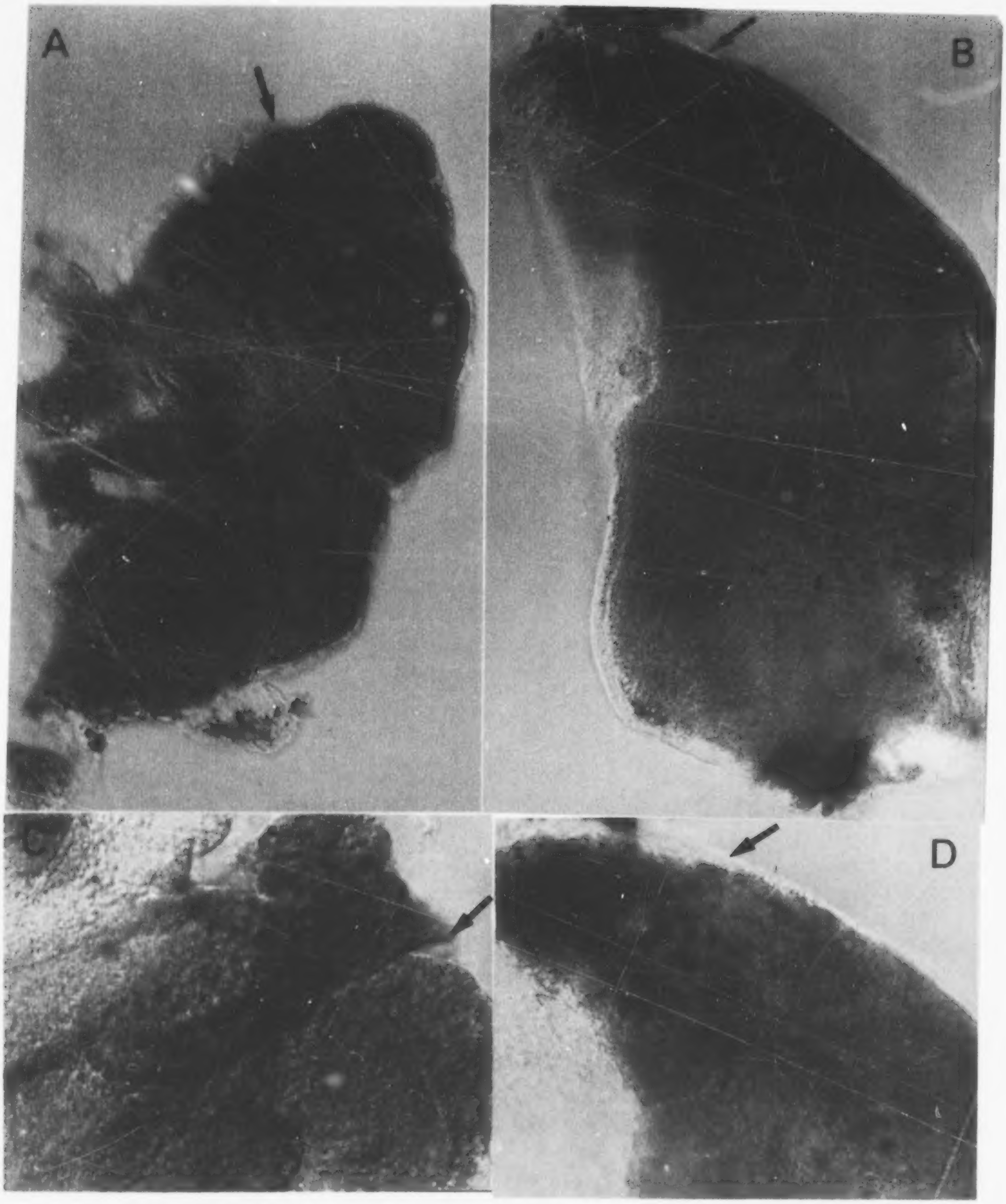

A
B
C
D

Table 4: Incidence of ectopic of *lacZ* expression in disc fragments cultured in vivo[a].

control *lacZ* staining pattern	class of insertion class I	class II	class III	class IV	Total
no expression	2/4	12/45	1/6	0/14	15/69
expression at morphogenetic furrow	0/1	1/4	-	-	1/5
neurogenic expression	0*/1	0*/4	-	-	0*/5
compartment boundary expression	2/2	5/5	-	-	7/7
Total	4/8	18/58	1/6	0/14	23/86

[a](Strains with ectopic expression in cultured fragments / total strains tested) is indicated for each category.

*these lines were difficult to interpret due to the complex control expression patterns

Table 5: Number of cultured disc fragments sampled from lines with no control expression pattern.

Lines	line scored as positive/negative	number of fragments recovered 1-5	6-10	>10	Total
all lines	total	49	18	2	69
	scored as positive	6	8	1	15
	scored as negative	43	10	1	54
class I	total	2	2	0	4
	scored as positive	2	0	0	2
	scored as negative	0	2	0	2
class II	total	32	12	1	45
	scored as positive	3	8	1	12
	scored as negative	29	4	0	33
class III	total	4	2	0	6
	scored as positive	1	0	0	1
	scored as negative	3	2	0	5
class IV	total	11	2	1	14
	scored as positive	0	0	0	0
	scored as negative	11	2	1	14

III.3.2 Enhancer-trap insertions expressed in control imaginal discs

Some insertions with patterned disc expression were also tested. Fifteen of sixteen lines with expression associated with a compartment boundary were assigned to classes I or II. This strong association with the target classes as well as the experimental results and theoretical considerations implicating compartments and compartment boundaries in disc patterning (see introduction for references) suggested genes in this class might play an important role in regeneration. As well, the role of cell-cell signalling during neural development and ommatidial assembly made insertions expressed in neurogenic patterns or near the morphogenetic furrow of the eye imaginal disc appealing candidates.

Preliminary examination of AD-55 "06" wing disc fragments suggested that there was ectopic expression associated with the healed wound. This fragment can heal somewhat irregularly and the cut runs through the control expression along the a/p boundary, making a straightforward interpretation difficult. A v-shaped cut, which yields a bi-lobed ventral fragment, was therefore attempted. This gave a fragment similar to the B-fragment of Dale and Bownes (1985), and was found to heal in a manner which was easier to interpret (I call this the "A/P fragment, see Figure 10). A/P-fragments from six insertions with expression along the anterior/posterior compartment boundary (AD-55, D-46, E-32, H-39, H-44, H-87) were cultured in adult female hosts. All six lines stained at the site of wound healing. Figure 11 shows an example of two such fragments from the insertion H-39. The original expression remaining in the ventral portion of the fragments serves as a convenient positional marker. At higher magnification (see Figure 11 D), new staining can be observed in a small number of cells (<50) on both sides of an obvious morphological discontinuity which was interpreted to be the site of wound healing.

Ectopic expression in different lines was found to be both symmetrically (D-46, H-39, H-44, H-87, and AD-55) and asymmetrically (E-32 and AD-55) distributed about the site of wound healing, possibly indicating different requirements for gene expression or perhaps different stages of wound healing. The insertion E-32 was always found to be expressed on only one side of the wound (see Figure 12 B, C), however, AD-55 was found to be expressed on one or both sides of the wound in different fragments recovered (see Figure 13). In all lines but AD-55 there were examples where the ectopic expression was found to be continuous with the original expression in the ventral portion of the fragment (see Figure 12 B, C). This may represent the re-establishment of a continuous compartment boundary during the early stages of wound healing. AD-55 might not be expected to show such a pattern as it is expressed discontinuously along the boundary in control discs (see Figure 13 A).

Certain fragments suggested that the ectopic staining in A/P-fragments in a/p boundary lines might be dependent on confrontation of cells from both the anterior and posterior compartments at wound healing. Staining was not observed in fragments which had not healed or where healing had only occurred between cells of the same compartmental origin leading to duplicating blastemas (Dale and Bownes, 1985) (see Figures 13 B, C, D) This hypothesis was directly tested for four lines (E-32, H-39, H-44 and AD-55) by cutting the

Figure 10: Healing patterns of the two cuts used to produce fragments from insertions expressed along to anterior-posterior compartment boundary. With the A/P cut, cells from both compartmental commitments come into contact during wound healing. The P cut produces a fragment which consists of only posterior compartment cells.

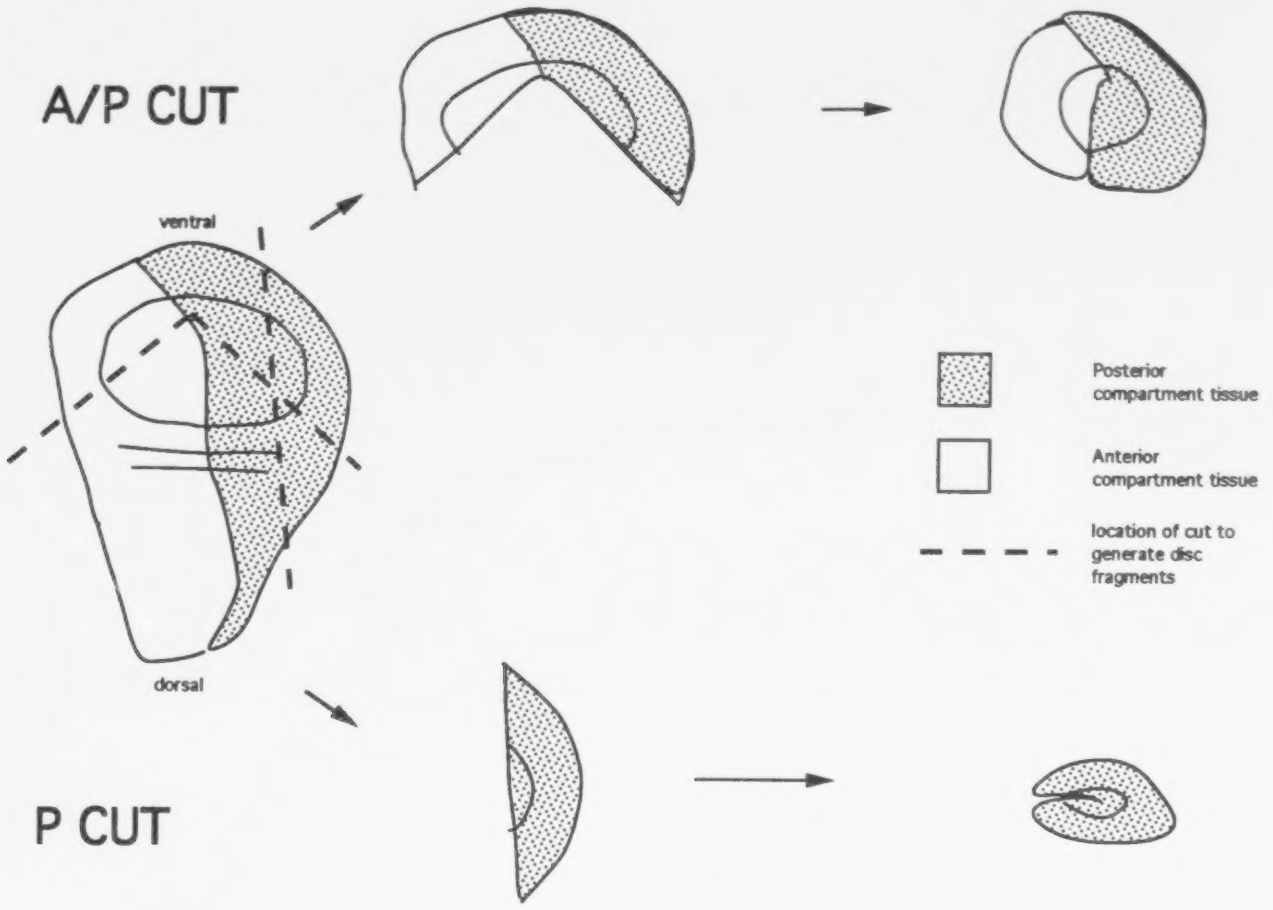

A/P CUT
ventral
dorsal
P CUT
Posterior compartment tissue
Anterior compartment tissue
location of cut to generate disc fragments

Figure 11: Ectopic *lacZ* expression in cultured H-39 A/P wing fragments. A: Control X-gal staining pattern in H-39 wing disc. The approximate locations of the cut to produce the A/P fragment are indicated (-------). Note the prominent expression at the a/p boundary and to the right, the weak expression in the posterior compartment. B and C: H-39 A/P fragments cultured in vivo for 24 h. The ectopic expression is found in cells on both sides of the healed wound (arrows). The location of the original staining is indicated (O). D: Higher magnification of the wound site of the fragment in panel B.

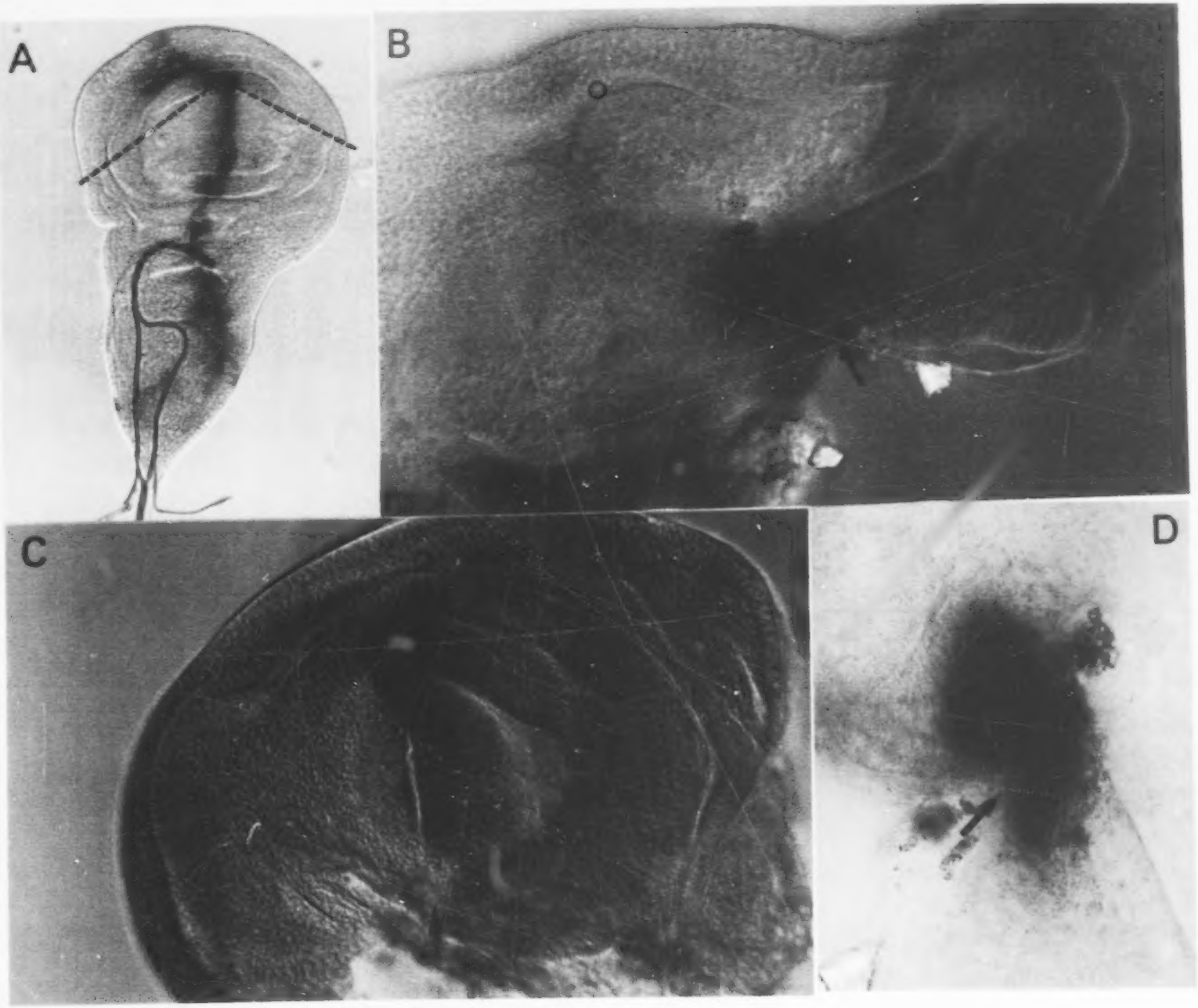

A
B
C
D

Figure 12: Ectopic *lacZ* expression in cultured E-32 A/P wing fragments. A: E-32 wing disc control X-gal staining pattern. The approximate locations of the cut to produce the A/P fragment are indicated (-------) as are the approximate locations of the cells which interact in the healed wound (a,p), and the position of the original staining (O) which remains in the fragment. B and C: E-32 A/P fragments after 24 hours in vivo culture. In both of these fragments, the staining is restricted to the "p" side of the healed wound (arrows). Note that in panel B. the stripe of staining bifurcates near the apex of the wound.

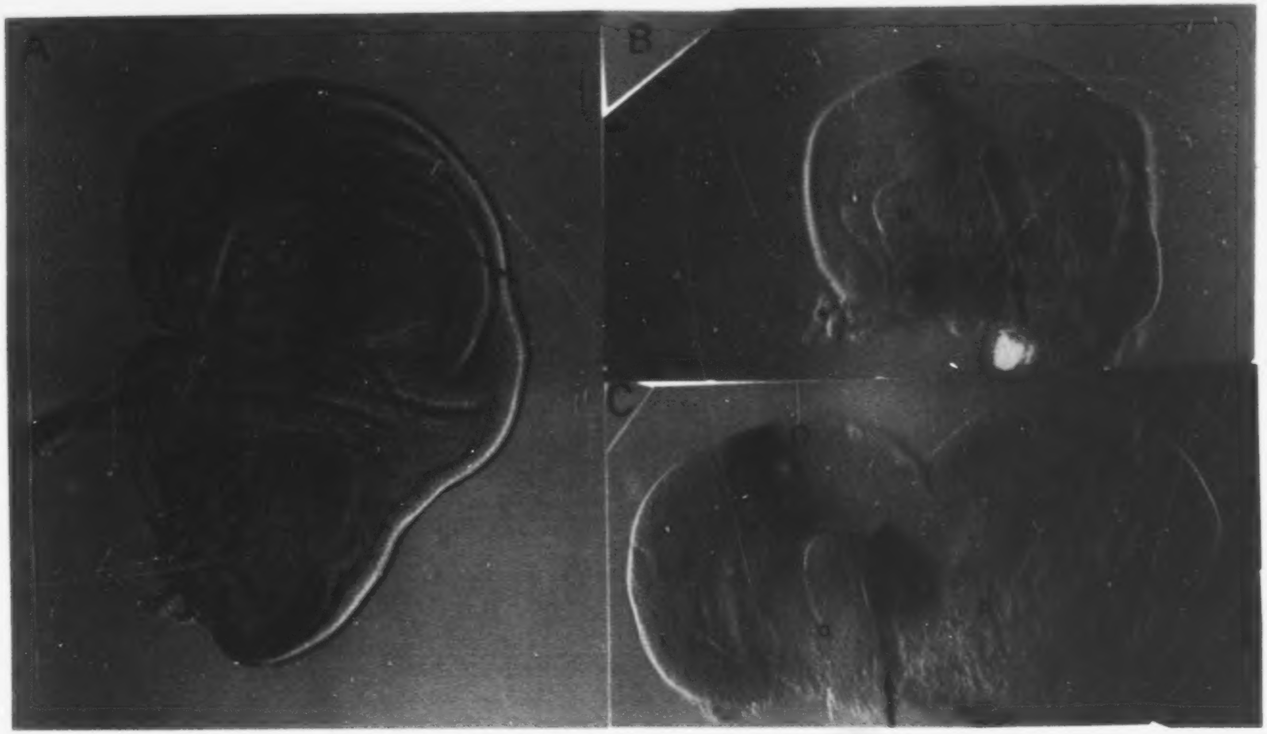
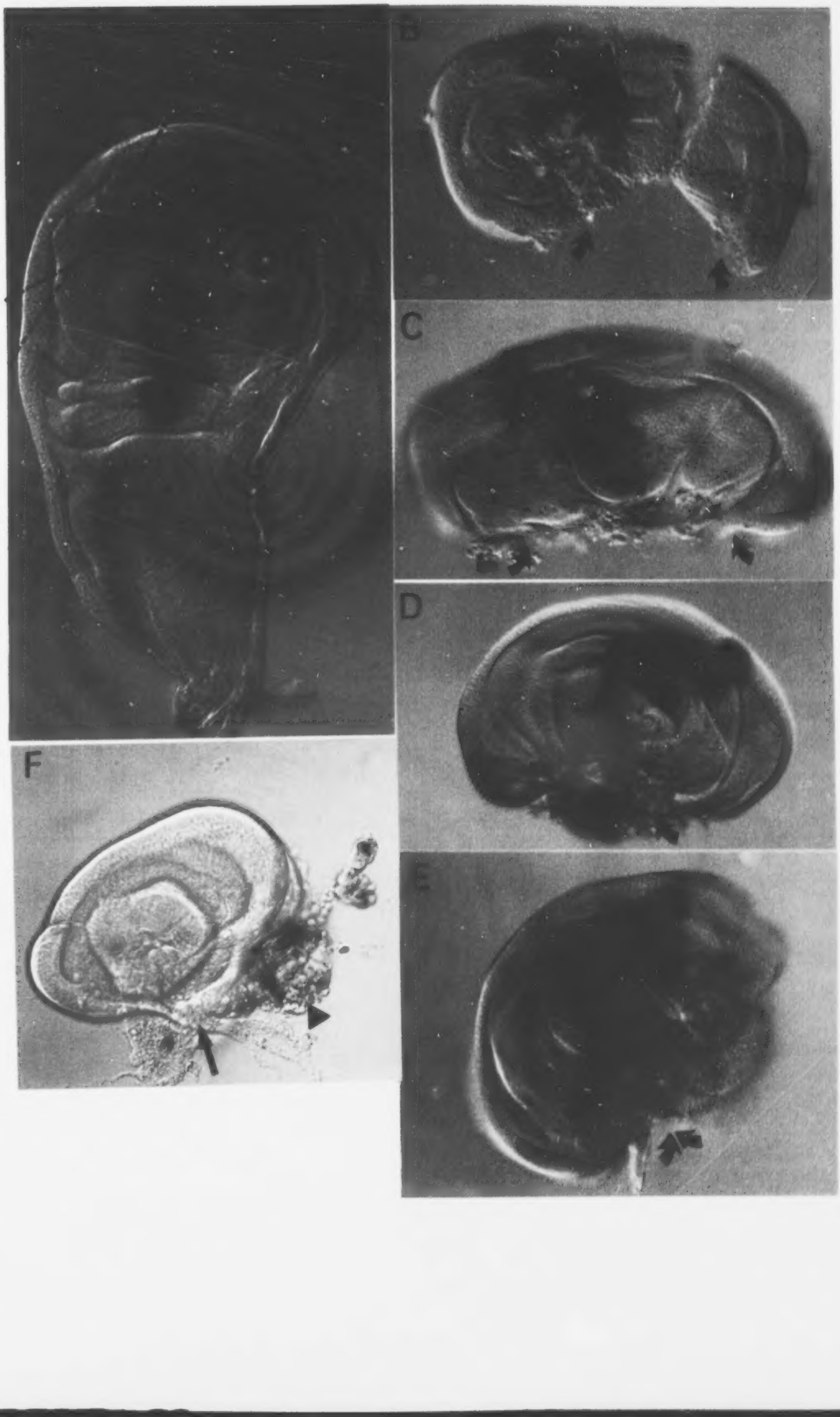

A
B
C

Figure 13: Ectopic *lacZ* expression in cultured AD-55 A/P and P wing fragments. A: Control X-gal staining pattern in an AD-55 wing disc. The approximate locations of the cut to produce the A/P and P fragments are indicated (-------) B-E: AD-55 A/P fragments cultured in vivo for 24 hours exhibiting different extents of healing, indicated by the arrows. Unhealed fragments (C) partially healed fragments (D), and fragments where the A and P lobes of the fragment heal back onto themselves (i.e.left hand lobe of fragment B) do not exhibit ectopic staining in the disc epithelium. E: P fragment cultured in vivo for 3 days. There is no staining in this fragment in the disc epithelium Strong ectopic staining is only observed where cells from the anterior and posterior compartment come into contact (as in E). There is staining in adhering material (triangle). This is not due to the enhancer-trap as this type of staining was also occasionally observed in cultured Canton-S fragments.


B
C
D
E
F

discs to produce a fragment which contains only posterior compartment tissue (Figure 10 and Figure 13 F). These fragments were cultured for as long four days with no induction of ectopic staining (see Table 6). These results indicate that the enhancer-trap expression in these lines during regeneration must be dependent on cell communication to assess the original compartmental states of neighboring cells in the regeneration blastema.

Some insertions from other classes with control imaginal disc expression were also tested using the cut shown in Figure 8. One insertion with expression along the dorsal-ventral wing margin (B-82) was observed to have ectopic expression in cultured disc fragments. It was, however, not determined whether or not the expression was dependent on confrontation of tissue from the dorsal and ventral compartments. Five insertions with control expression patterns suggestive of a role in neurogenesis were examined, but it was found that the complex control patterns exhibited by these insertions made unambiguous interpretation of these insertions impractical. One insertion (C-07) of five with control expression folowing the morphogenetic furrow in the eye-antennal disc had ectopic expression in cultured wing disc fragments. This insertion was also expressed in polar follicle cells in the ovary (see Figure 14).

The proportion of lines showing ectopic expression in cultured disc fragments supports the assumptions made in the design of the initial screen. Class I included the highest proportion of positives (4/8) followed by Class II (19/58), Class III (1/6), and Class IV (0/14).

III.4 Summary

From a sample of 826 PZ enhancer-trap insertions, 23 were directly demonstrated to be expressed in regenerating disc fragments. All but one of these insertions were in classes I or II, the most likely target classes defined by the screens. If the sample of lines tested by *in vivo* culture of disc fragments is representative, as many as 74 of the 826 lines may be ectopically expressed in the regeneration blastema. Table 7 summarizes the disc cutting and developmental expression profiles of these insertions.

Table 6: Cultured disc fragments from insertions expressed at compartment boundaries.

line	06 fragment	A/P-fragment	P-fragment
B-82	3/7	nd	nd
D-46	nd	6/13	nd
E-32	nd	12/21	0/2
H-39	nd	9/16	0/3
H-44	nd	4/11	0/1
H-87	nd	5/11	nd
AD-55	2*/3	11/14	0/4

The "06" fragment and the "A/P" and "P" fragments are indicated in Figures 8 and 10, respectively.

Figures are presented as (total fragments with ectopic staining)/(total fragments recovered).

*indicates that the expression scored as ectopic was ambiguous.

Figure 14: *lacZ* expression patterns in C-07. A: Intact X-gal stained C-07 wing disc showing approximate position of cut (dashed lines) generating a notum fragment (n) for in vivo culture. B: C-07 notum fragment stained after culture in adult female host. *lacZ* expression is localized (arrow) at presumed site of wound healing. Open arrows in A show folding of cut edge which would juxtapose normally non-expressing cells (near the dots) at the site of expression evident in B (arrow). C: Untreated X-gal stained C-07 eye-antenna disc showing control expression in distal antenna (a) and and in eye disc (e) posterior to the morphogenetic furrow (arrows), where assembly of ommatidia is visible. D: C-07 expression in adult ovary. X-gal staining is evident in the germarium (upward arrow), in polar follicle cells (downward arrows), and also in columnar follicle cells (white arrows) at late stages of oogenesis. This figure is reproduced from Brook et al. (1993) with the permission of The Company of Biologists, Ltd.

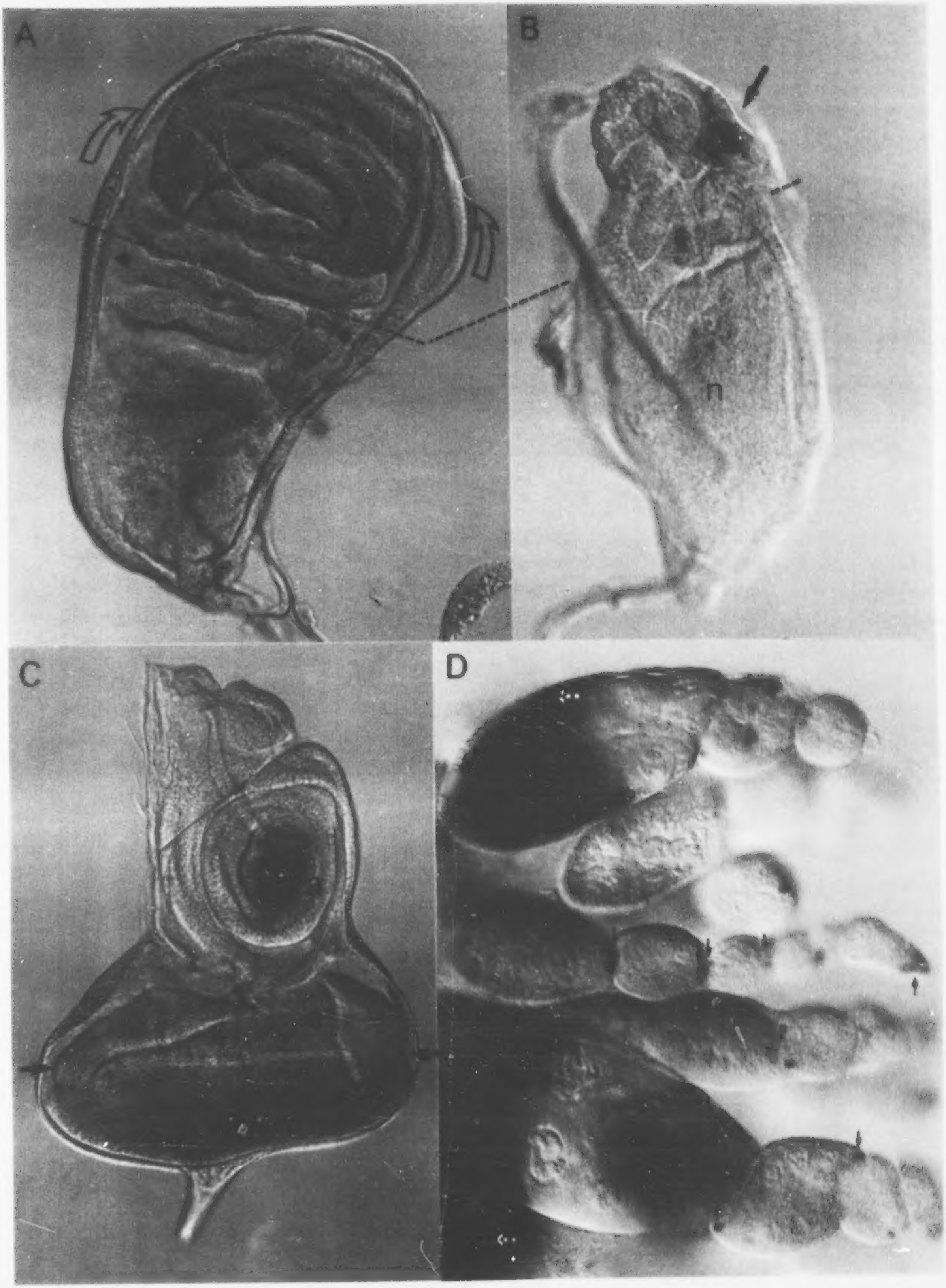

A
B
n
C
D

Table 7: *lacZ* expression profiles of insertions ectopically activated in cultured wing disc fragments.

Strain (Class)	wing disc staining			ovary pattern [stage][b]	embryonic pattern
	control	fragment[a]			
B-17 (II)	none	wound-heal		NC [10]	CNS
C-39 (III)	none	wound-heal		NC [10]	CNS/PNS
C-92 (II)	none	wound-heal		none	midline CNS
D-42 (I)	none	wound-heal		none	lateral epidermis (SEG)
E-17 (II)	none	wound-heal		germarium	CNS/PNS
E-34 (II)	none	wound-heal		none	weak
E-91 (II)	none	wound-heal		none	CF, PNS
F-22 (II)	none	wound-heal		none	weak
F-45 (II)	none	wound-heal		none	none
G-45 (II)	none	wound-heal	(see Figure 9)	none	midline CNS
H-21 (I)	none	wound-heal		NC [10]	CF, epidermis (SEG)
C-76 (II)	none	general		FC, columnar [10]	midgut, Malphigian tubules
E-37 (II)	none	general	(see Figure 9)	none	none
E-60 (II)	none	general		FC, posterior columnar [8]	weak
F-36 (II)	none	general		none	CF, dorsal epidermis
C-07 (II)	none[c]	wound-heal	(see Figure 14)	FC,polar [1],columnar [9]	CNS
B-82 (I)	d-v margin	wound-heal		FC [9]	ventral epidermis, PNS, hindgut
D-46 (II)	a/p boundary	wound-heal[d]		none	none
E-32 (II)	a/p boundary	wound-heal[d]	(see Figure 12)	germarium, NC	lateral epidermis(SEG), dorsal epidermis
H-39 (II)	a/p boundary	wound-heal[d]	(see Figure 11)	FC [12]	CF, PNS, CNS, epidermis
H-44 (II)	a/p boundary	wound-heal[d]		none	none
H-87 (II)	a/p boundary	wound-heal[d]		FC, squamous [9]	amnioserosa, dorsal epidermis(SEG)
AD-55 (I)	a/p boundary	wound-heal[d]	(see Figure 13)	FC, polar [8], border cells	mesodermal

[a] "wound heal" indicates that ectopic expression in culturede disc fragments is restricted to the site of wound healing. "general" means ectopic staining away from the wound as well as expression at the wound-heal.

[b] Numbers in brackets refer to first stage (King, 1970) when expression is detected.

[c] C-07 is expressed posterior to the morphogenetic furrow in eye disc, and in the tibia/AIII regions of the leg and antenna discs.

[d] Expression at the healed wound for these lines was conditional on contact between anterior compartment cells and posterior compartment cells during wound healing

Abbreviations: NC: nurse cells; FC: follicle cells; CNS: part or all of central nervous system; PNS: part or all of peripheral nervous system; CF: cephalic furrow; SEG: segmental epidermal expression.

IV. GENETIC AND MOLECULAR ANALYSES OF ENHANCER-TRAPS EXPRESSED IN REGENERATING IMAGINAL DISCS.

Enhancer-trap elements identify patterns of transcriptional regulation acting at the point of insertion in the genome. These reporter gene expression patterns may reflect all or only some of the enhancers controlling a particular gene or genes close to the site of insertion. Ultimately, a complete characterization of all the transcription units near an insertion may be necessary to determine which transcript(s) an element reports. This chapter describes a preliminary genetic and molecular characterization of 7 Class I and Class II enhancer-traps found to be ectopically expressed in regenerating imaginal discs. This is the first step in a genetic analysis of imaginal disc regeneration – to determine what kind of genes are being expressed in cells of the regeneration blastema. Four insertions are identified based on mutant phenotype (B-82, C-92), expression pattern (E-32, AD-55), and cytogenetic location. Three further insertions which apparently identify novel loci but do cause mutant phenotypes were analyzed because they had striking expression patterns in cultured disc fragments (G-45, H-39) or in one case (H-15), an expression pattern which suggests a role in imaginal disc pattern formation. Enhancer-traps identifying new loci offer several approaches for subsequent analysis, including generation of mutations by imprecise P-element excision and cloning of the genomic insertion sites by plasmid rescue.

IV.1 Identification of loci by homozygous lethal phenotype

The 826 enhancer-trap strains included 139 (17%) which were homozygous lethal. This value is similar to frequencies in the order of 10-20% reported in similar screens (Cooley et al., 1988, Bier et al., 1989; Bellen et al., 1989). Forty-one of the two hundred lines from classes I and II were homozygous lethal. The lethal stages of these lines were determined as described in the Methods and are presented in Table 8. All stages of lethality (i.e. embryonic, larval, or pupal) were observed in both classes and no single stage predominated. Although the phenotypes were not exhaustively studied, two insertions among the 23 expressed in regenerating discs were associated with striking embryonic lethal phenotypes.

IV.1.1 B-82 is an insertion in the *crumbs* locus

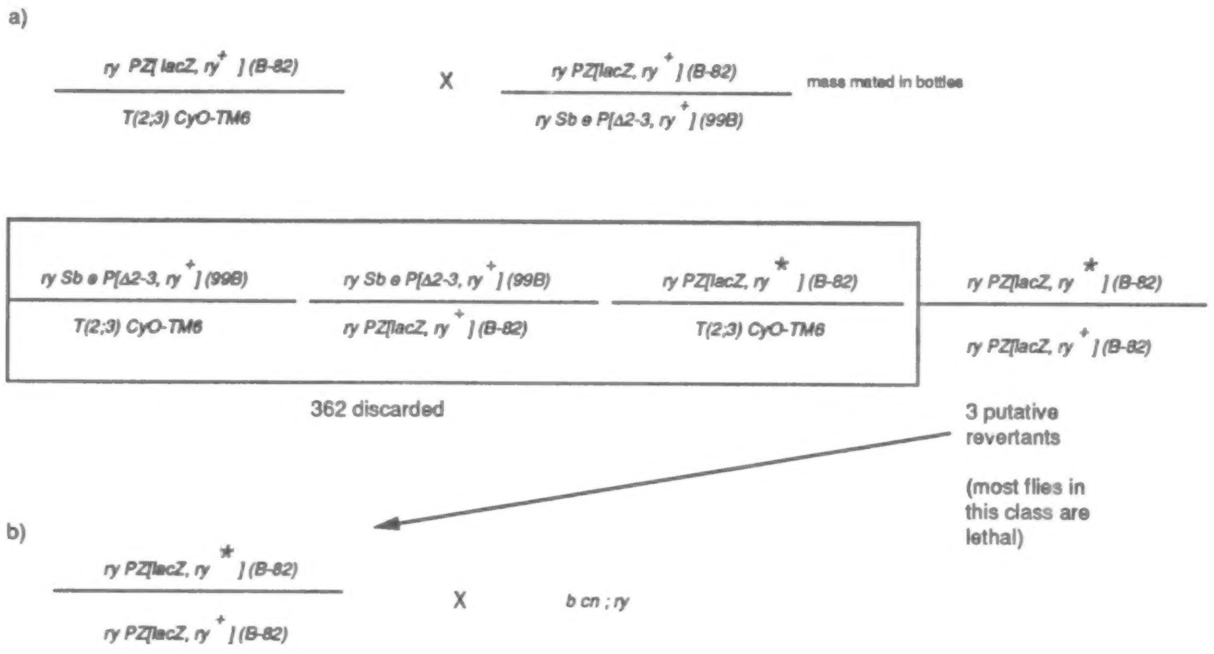
B-82 is a class I, embryonic lethal, third chromosome insertion (33/142, 25% of embryos fail to hatch). The homozygous lethal embryos were found to have a very degenerate cuticle pattern. The insertion causes this phenotype as it was possible to revert the lethality by Δ2-3 induced P-element excision, demonstrated by the simultaneous loss of the ry^+ marker of the PZ element (see Figure 15). B-82 was mapped to the third chromosome. A previously described mutation, *crumbs* (Jürgens et al., 1984) has a very similar phenotype to B-82 homozygotes and mapped to polytene bands 95F-96A (Tepass and Knust, 1990).

Table 8: Lethal stages of homozygous lethal insertions

	class					
stage	I	II	III	IV	V	all classes
embryonic	2	3	1	1	1	8
1st larval instar*	0	6	1	0	0	7
2nd larval instar*	2	3	0	0	0	5
3rd larval instar*	1	2	0	1	0	4
pupa	3	2	0	1	0	6
pharate adult	0	8	0	0	0	8
not determined	1	8	2	21	69	101
total	9	32	4	24	70	139

*larval lethality is estimated based on the oldest Tb^+ larva(e) observed (see Methods)

Figure 15: Generation of revertants of the lethal P-element insertion B-82


a)
ry PZ[lacZ, ry+] (B-82)
T(2;3) CyO-TM6
X
ry PZ[lacZ, ry +] (B-82)
ry Sb e P[Δ2-3, ry +] (99B)
mass mated in bottles
ry Sb e P[Δ2-3, ry +] (99B)
T(2;3) CyO-TM6
ry Sb e P[Δ2-3, ry +] (99B)
ry PZ[lacZ, ry +] (B-82)
ry PZ[lacZ, ry *] (B-82)
T(2;3) CyO-TM6
ry PZ[lacZ, ry *] (B-82)
ry PZ[lacZ, ry +] (B-82)
362 discarded
3 putative revertants
(most flies in this class are lethal)
b)
ry PZ[lacZ, ry *] (B-82)
ry PZ[lacZ, ry +] (B-82)
X
b cn ; ry

	rosy +	rosy
revertant 1	37	13
revertant 2	10	12
revertant 3	18	11

All putative revertants segregated for the rosy marker implying that the P-element is lost simultaneously with the reversion of the lethality

In situ hybridization of the Carnegie-20 plasmid (Rubin and Spradling, 1983) (which contains P-element sequences as well as sequences from the *white* and the *rosy+* loci) to polytene chromosomes from the B-82 strain showed hybridization to the *white* (3C)and *rosy* (87EF) loci and ectopic hybridization within polytene location 95-96 (not shown). Furthermore, B-82 failed to complement a *crumbs* deficiency (Df(3R)crb^{S87-4}) and a *crumbs* point mutation (crb^{MA22}) and the transheterozygotes also displayed the *crb* phenotype. The B-82 embryonic expression pattern is very similar to the reported expression for *crb* (Tepass et al., 1990). A weak, uniform expression pattern is reported for the *crb* protein in all imaginal discs. The *lacZ* expression pattern of B-82 in leg, haltere, and eye-antennal discs is quite similar to that of the *crb* protein, but the wing discs is characterized by prominent expression along the d/v margin. These results indicate that B-82 is an insertion in the *crb* locus, a gene which encodes an EGF-repeat containing protein.

IV.1.2 The C-92 insertion is a larval lethal associated with a maternal effect lethal with a *bicaudal* like phenotype

The insertion, C-92, is a class II insertion on a homozygous lethal second chromosome. Although the terminal lethal phase was larval, a variable frequency of embryonic lethality was also observed. The unhatched embryos often displayed a headless phenotype, or variable expression of a mirror image double abdomen phenotype. A series of experiments was undertaken to determine if the P-element was the cause of these phenotypes.

The C-92 insertion is the cause of the larval lethal phenotype as it is possible to revert the lethality simultaneously with loss of the *rosy+* marker of the element (Figure 16). Furthermore, the insertion site was mapped by *in situ* hybridization to polytene band 42A, near the base of the right arm of the second chromosome. C-92 complements In(2R)$bw^{vde2L}Cy^{2R}$ (which contains a deficiency concomitant with the inversion) and Df(2R)nap^{8} which have distal break points in 42A2-7, but it fails to complement Df(2R)nap^{11} which has a distal breakpoint in 42A7-10 (Kernan et al., 1991). All three deficiencies have proximal breakpoints in section 41. Thus the lethality was mapped to a discrete interval with endpoints 42A2-7 and 42A7-10. There are no published mutants in this interval (Lindsley and Zimm, 1992), so the C-92 insertion identifies a novel zygotic lethal gene.

The double abdomen phenotype is a maternal effect as it was only seen when the C-92 chromosome was present in the maternal genome. The proportion of embryos with the *bicaudal*-like phenotype was found to vary substantially in repeated crosses suggesting that its expression is sensitive to non-genetic factors such as culture conditions or maternal age. Similar variability has been reported previously for strains producing double abdomen embryos (Nüsslein-Volhard 1977; Mohler and Wieschaus 1986). The double abdomen phenotype was never detected among dead embryos from stocks homozygous for chromosomes reverted for the P-element induced zygotic lethality. This suggests that the insertion may also be responsible for the maternal effect. However, the variable expression and unrepeatability of the

Figure 16: Generation of revertants of the lethal P-element insertion C-92

a)

$$\frac{b\ PZ[\,lacZ,\ ry^{+}]\ (C\text{-}92)}{CyO}\ \ \frac{ry}{ry}\ \times\ \frac{b\ PZ[\,lacZ,\ ry^{+}]\ (C\text{-}92)}{CyO}\ \ \frac{ry\ Sb\ e\ \ P[\ \Delta 2\text{-}3,\ ry^{+}]\,(99B)}{ry}$$

1852 Cy or Cy, Sb flies discdared

$$\frac{b\ PZ[\,lacZ,\ ry^{*}]\ (C\text{-}92)}{b\ PZ[\,lacZ,\ ry^{+}]\ (C\text{-}92)}\ \ \frac{ry\ Sb\ e\ \ P[\ \Delta 2\text{-}3,\ ry^{+}]\,(99B)}{ry}$$

5 putative Sb revertants discared

$$\frac{b\ PZ[\,lacZ,\ ry^{*}]\ (C\text{-}92)}{b\ PZ[\,lacZ,\ ry^{+}]\ (C\text{-}92)}\ \ \frac{ry}{ry}$$

8 putative
revertants

(most flies
in this class
are lethal)

b)

$$\frac{b\ PZ[\,lacZ,\ ry^{*}]\ (C\text{-}92)}{b\ PZ[\,lacZ,\ ry^{+}]\ (C\text{-}92)}\ \ \frac{ry}{ry}\ \times\ \ \frac{Sp}{CyO}\ \ \frac{ry}{ry}$$

	Sp ry	Cy ry	Sp ry^{+}	Cy ry^{+}
revertant 1	44	69	90	86
revertant 2	57	81	102	112
revertant 3	38	82	61	77
revertant 4	46	62	72	101
revertant 5	47	54	61	67

5/6 putative revertants segregated for the rosy marker implying that the P-element is lost simultaneously with the reversion of the lethality. one revertant did not segregate for ry, implying that the marker was retained within the genome. The other two were not tested as they were non-virgin females.

double abdomen phenotype seen in the original C-92 stock (i.e 0-25%) makes it impossible to be certain that the lack of expression is due to reversion of the zygotic lethal mutation or if the maternal phenotype is simply suppressed by modifiers in the revertant stocks.

No *lacZ* expression could be detected in C-92 ovaries (see Table 7). Furthermore, saturation screens for maternal effect lethals on the second chromosome have failed to detect any maternal lethals which map near C-92 (Schüpbach and Wieschaus, 1989). Although deficiencies uncovering the lethality of C-92 have not been tested for a haplo-insufficient bicaudal effect, it seems likely that if the C-92 insertion is the cause of the double abdomen phenotype, it is via a gain-of-function mutation rather than loss of function required during oogenesis. Neomorphic mutations causing double abdomen phenotypes have been reported for genes whose loss-of-function phenotypes have no bearing on oogenesis or female fertility (Whittle, 1976; Grau and Simpson, 1987) Therefore, while it is potentially interesting that the insertion may cause a bicaudal-like phenotype, this may not reflect the normal function of the gene disrupted by the C-92 insertion.

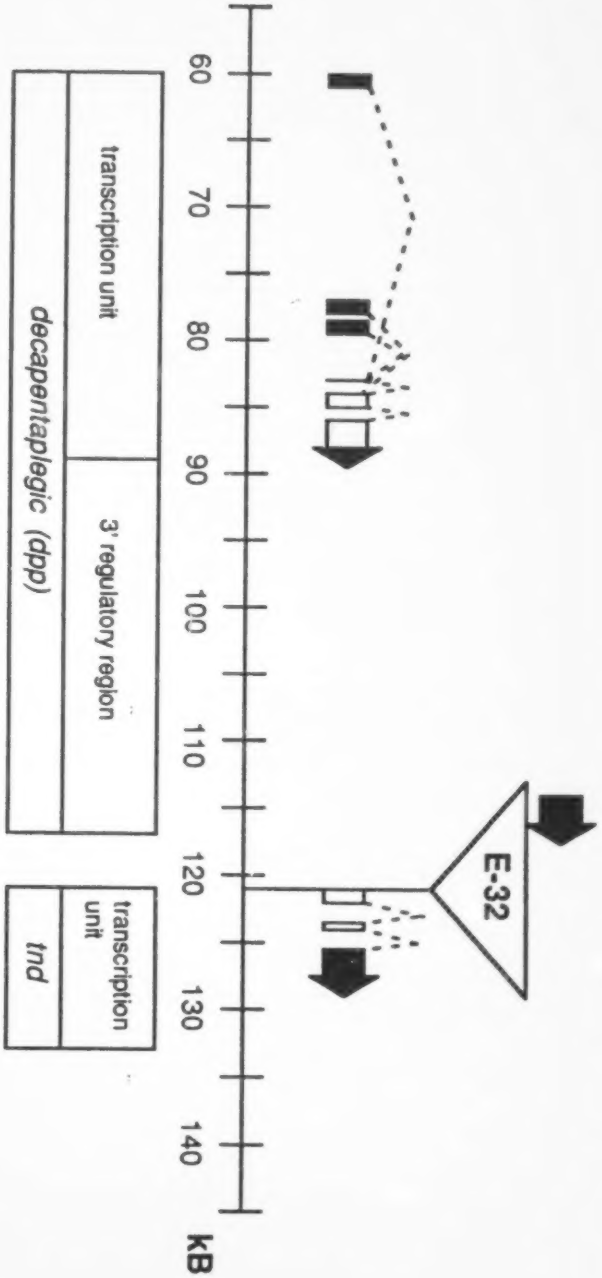
IV.2 Identification of loci by cytogenetic location and expression pattern.

The cytogenetic location of several insertions having interesting expression patterns and/or lethal phenotypes was determined by *in situ* hybridization to polytene chromosomes using enhancer-trap specific sequences as probes. The results are summarized at the end of this chapter in Table 11. The combination of insertion site and expression pattern proved very useful in identifying two loci.

E-32, a class II insertion, was found to be inserted at polytene location 22F, the location of the gene, *decapentaplegic* (*dpp*) (Spencer et al., 1982) . *dpp* encodes a secreted protein which belongs to the TGF-β superfamily (Padgett et al., 1987). Like *dpp*, E-32 is expressed in a narrow stripe close to the anterior-posterior compartment boundary in imaginal discs (Figure 6 G, Figure 12 A) and in a segmentally repeated pattern in the ventrolateral ectoderm of the embryo. Plasmid rescue of the sequences flanking the E-32 insertion was done by Dr. Ron Blackman, University of Illinois. Blackman determined that E-32 was inserted adjacent to the *dpp* locus, in the 5' untranslated leader of an adjacent transcription unit, *transcript-near-decapentaplegic* (*tnd*) (see Figure 17). *tnd* is not normally expressed in imaginal discs or in the embryo (R. Blackman pers. comm.). E-32 is also expressed in the nurse cells, oocyte (see Table 7), testes, and larval gonads which are sites of expression of the *tnd* transcript and not the *dpp* transcript (Dr. R. Blackman, pers. comm.). This indicates that E-32 must report both *dpp* and *tnd* expression.

AD-55 was found to be inserted in polytene location 60CD. It is expressed in the same pattern as an enhancer-trap from that region called LF-06, which was previously isolated by Dr S. Eaton (pers. comm.). Southern blot analysis indicated that the AD-55 insertion changed the size of the same

Figure 17: Insertion of E-32 in the *dpp tnd* region. The enhancer-trap, E-32, which reports both the *dpp* and *tnd* transcription patterns, is inserted in the 5' untranslated leader of the *tnd* transcription unit (R. Blackman, pers. comm.). The diagram is after St. Johnston et al. (1990) and R. Blackman (unpublished).


60
70
80
90
100
110
120
130
140
kB
E-32
transcription unit
3' regulatory region
decapentaplegic (dpp)
transcription unit
tnd

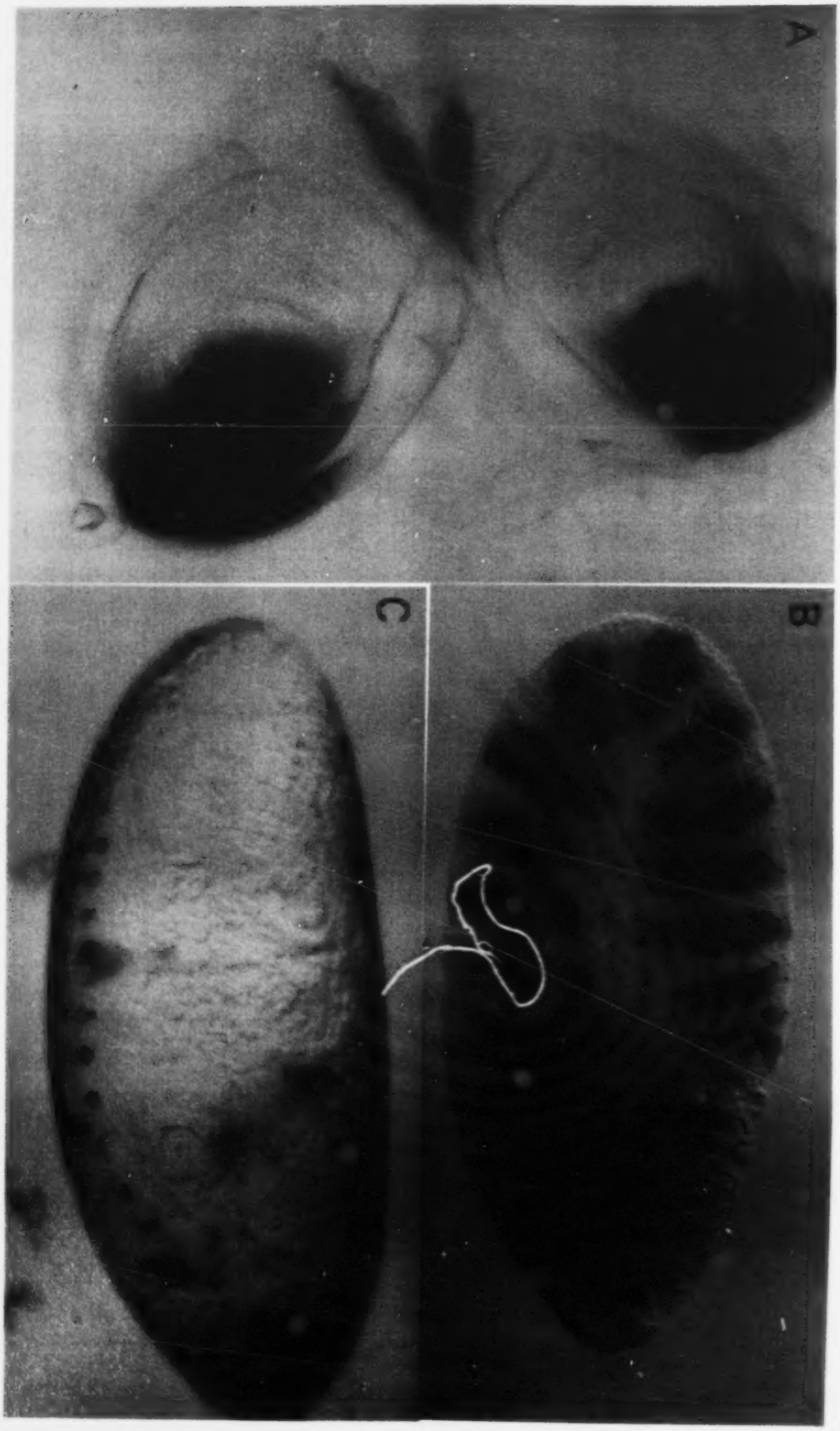
restriction fragment as the LF06 enhancer-trap indicating that they both report the same gene. A cDNA from the region which is expressed in a similar pattern to both enhancer-traps encodes a novel protein (Dr S. Eaton, pers. comm.).

As indicated in the chapter summary (Table 11) several of the enhancer-traps localized by *in situ* hybridization had no mutant phenotypes and no nearby, previously characterized genes with corresponding expression patterns. Three such insertions are G-45, H-15, and H-39. G-45 is a class II insertion found to be inserted in 100E, with expression in the embryonic CNS (see Figure 18 C). There are no described loci in that region with a corresponding pattern of expression. The insertion H-15 (class II) is expressed in segmental stripes in the embryonic ectoderm and in the ventral portions of the antennal and leg discs (see Figure 18 A, B). This pattern is similar to those described for several segment polarity genes. H-15 was found to be inserted in band 25F where no segment polarity genes have been identified. H-39, another class II insertion, was localized to polytene region 75DE and was expressed along the anterior- posterior boundary in all imaginal discs (Figure 11 A). There are no cloned genes in this region which are expressed in a similar pattern and very few mutants have been identifed in this interval. Although it was in class V, and not selected in the screen, H-09 was localized because of the similarity of its expression to the segment polarity gene *wingless* (van den Heuvel et al., 1989; Baker, 1988) (i.e Figure 6 ,G,H). It was found to be inserted not at the *wingless* locus, 28A, but rather in polytene region 72EF. No segment polarity genes have been identified in this region. Two other insertions, H-21 and F-45 were also localized on the polytene map but have not been characterized further.

IV.3 Characterization of novel enhancer-traps which do not cause mutant phenotypes

The remobilization of P-element insertions to create mutations in flanking genes has been reported for several loci. These examples include two basic approaches. In the first a P-insertion chromosome is further subjected to transposase produced by the introduction of the P[Δ2-3]99B element or by a dysgenic cross followed by screening for the desired phenotype (Tsubota et al., 1986; Salz et al., 1987). The second approach involves selecting first for loss of a marker gene in the P-element following exposure to Δ2-3, then screening the selected chromosomes for a phenotype either homozygous or over a deficiency (for examples see Fasano et al., 1991; Doe et al., 1991). Several assumptions must be made when attempting to induce a mutation in a gene reported by an aphenotypic enhancer-trap element. The principal assumption is that the gene can be mutated to a cause a phenotype. Enhancer-traps identify genes by transcriptional control not by loss of function; it is always a possibility that the gene identified by an enhancer-trap may have a redundant or trivial function, a function that is not essential for viability or fertility under the experimental conditions, or a subtle loss of function phenotype. In the context of this screen, it is possible that a gene might be dispensable for normal development but required in regeneration. The next important assumption concerns the position of the enhancer-trap relative to the gene it reports. There are situations where

Figure 18: *lacZ* expression patterns of H-15 and G-45. A: H-15 First leg discs with X-gal staining restricted to a ventral sector and in the membrane joining the two discs. B: H-15 extended germ band embryo immunostainined with anti-β-galactosidase showing segmental epidermal and neural staining. Arrows indicate expressing cells which may be imaginal disc primordia. C: X-gal staining of late stage G-45 embryo with expression in laterally paired cells in the CNS midline (arrow).


A
B
C

an enhancer-trap is separated by an intervening gene from the gene it reports (Bourgouin et al., 1992) or where the enhancer-trap is inserted at a considerable distance from the gene it reports. This is the case with E-32 (see Figure 17). In these situations, interpretation of the mutant phenotypes generated by flanking deletions may be confounded by the simultaneous deletion of several functions in addition to the function of interest. Thus an approach based on a parsimonious evaluation of the range of mutant phenotypes generated from the excision of an enhancer-trap may sometimes be misleading.

With these caveats in mind, I undertook the characterization of G-45, H-15, and H-39. Deficiencies uncovering the insertion sites for all three lines were available and all three lines had potentially significant expression patterns in the context of imaginal disc regeneration and/or pattern formation.

IV.3.1 Cloning of genomic sequences flanking the H-39 insertion

The genomic DNA flanking the H-39[PZ] insertion was cloned by Dr. W.R. Addison and Ms. L. Querengesser. The right flank of the H-39 insertion was cloned by plasmid rescue of a *Hpa* II fragment (see Figure 3). The plasmid was subcloned and restriction mapped. A restriction map of the plasmid is shown in Figure 19. A 1.8 kB *Hind* III fragment from pH-39-R was gel-purified and used as a probe for *in situ* hybridization to polytene chromosomes. It hybridized to 75D, the polytene region previously identified with a P-element probe as the site of the H-39 insertion, indicating that pH-39-R had sequences from the correct region of the genome (see Figure 20).

An EMBL-4 library made from Oregon-R genomic DNA (a gift of Dr. S. Tiong) was screened by L. Querengesser using the same 1.8 kB *Hind* III fragment. Several positives were rescreened twice in order to obtain pure phage cultures. DNA from five of the recombinant phage was purified. None of the five recombinant phage shared common restriction fragments when digested with *Eco* R I, suggesting that they were non-overlapping phage, perhaps sharing a common DNA sequence, but derived from different genomic locations. A Southern hybridization of the digested phage DNAs to a probe made from the 1.8 kB *Hind* III fragment showed that one of the phage, λ25, hybridized much more strongly to the probe than the other four. Only λ25 was further characterized.

In situ hybridization of the entire inserted region of λ25 to polytene chromosomes revealed more than 30 sites of hybridization suggesting that the cloned DNA includes a moderately repetitive sequence. *In situ* hybridization of the 5.4 kB *Eco* R I fragment in p25RL(19 B) to polytene chromosomes resulted in only one site of hybridization to polytene region 75D, indicating that λ25 also contains sequences flanking the H-39 insertion point.

Figure 19: A: Restriction map of H-39 insertion of flanking genomic DNA. Locations of the 1.8 kB *Hind* III polytene *in situ* probe and the 1.6 kB tissue *in situ* probe are indicated. B: Relationship of pH-39-R, λ25, and p25RL to the composite map. Figure courtesy of Dr. W.R. Addison

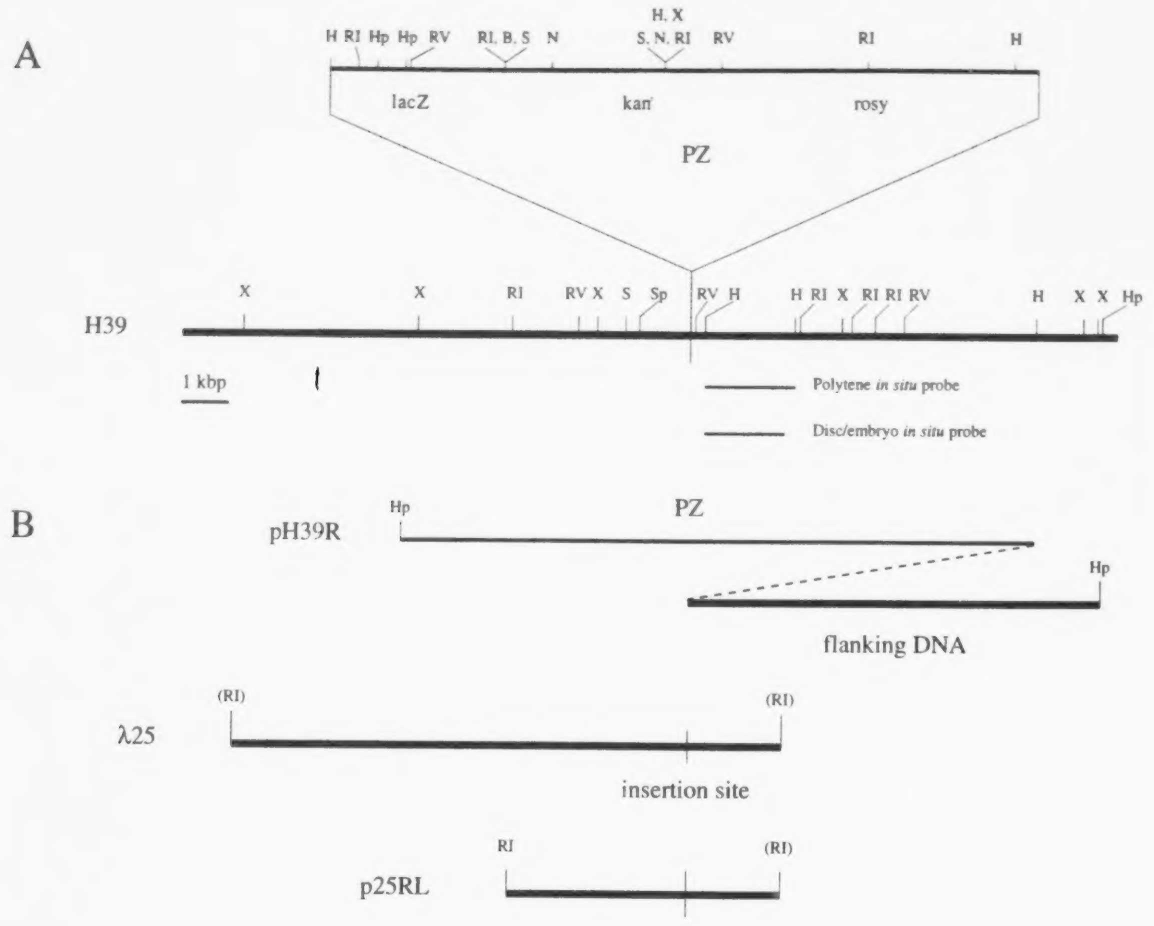
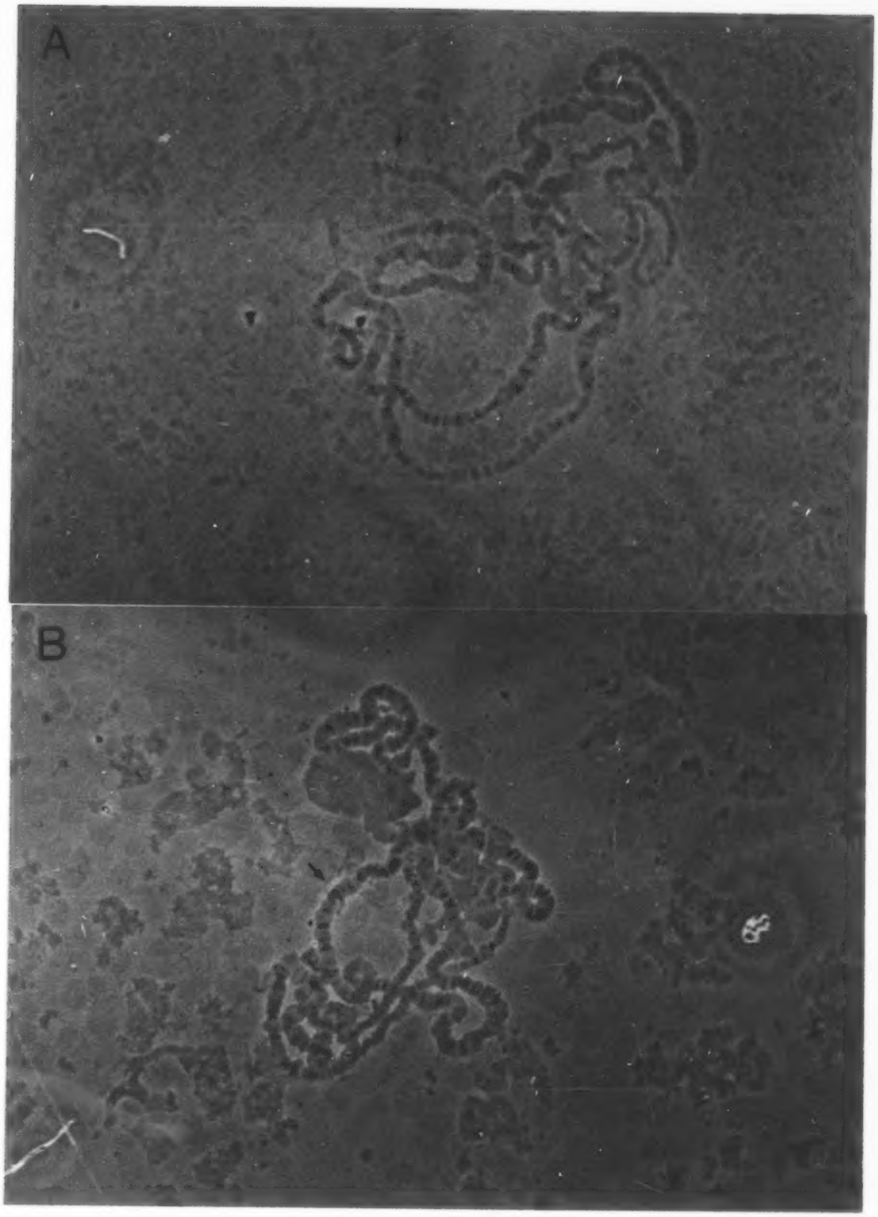

A
H RI Hp Hp RV
RI, B, S
N
H, X
S, N, RI
RV
RI
H
lacZ
kan'
rosy
PZ
H39
X
X
RI
RV X
S
Sp
RV
H
H RI
X
RI RI RV
H
X X Hp
1 kbp
Polytene *in situ* probe
Disc/embryo *in situ* probe
B
pH39R
Hp
PZ
Hp
flanking DNA
λ25
(RI)
(RI)
insertion site
p25RL
RI
(RI)

Figure 20: In situ hybridization of H-39 and Canton-S chromosomes. A: Hybridization of Carnegie 20 plasmid to H-39 chromosomes. Ectopic hybridization to 75DE indicated (arrow). Hybridization at *rosy* is not visible in this nucleus. (*in situ* hybridization was performed by Dr. S. Tiong). B: Hybridization of 1.8 kB *Hind* III fragment of p H-39-R to Canton-S chromosomes. Hybridization to 75DE indicated (arrow).


A
B

A map of the genomic DNA flanking the H-39 insertion point, derived from a composite map of pH-39-R and λ25 was prepared by Dr. W.R. Addison. This data is presented in Figure 19 A.

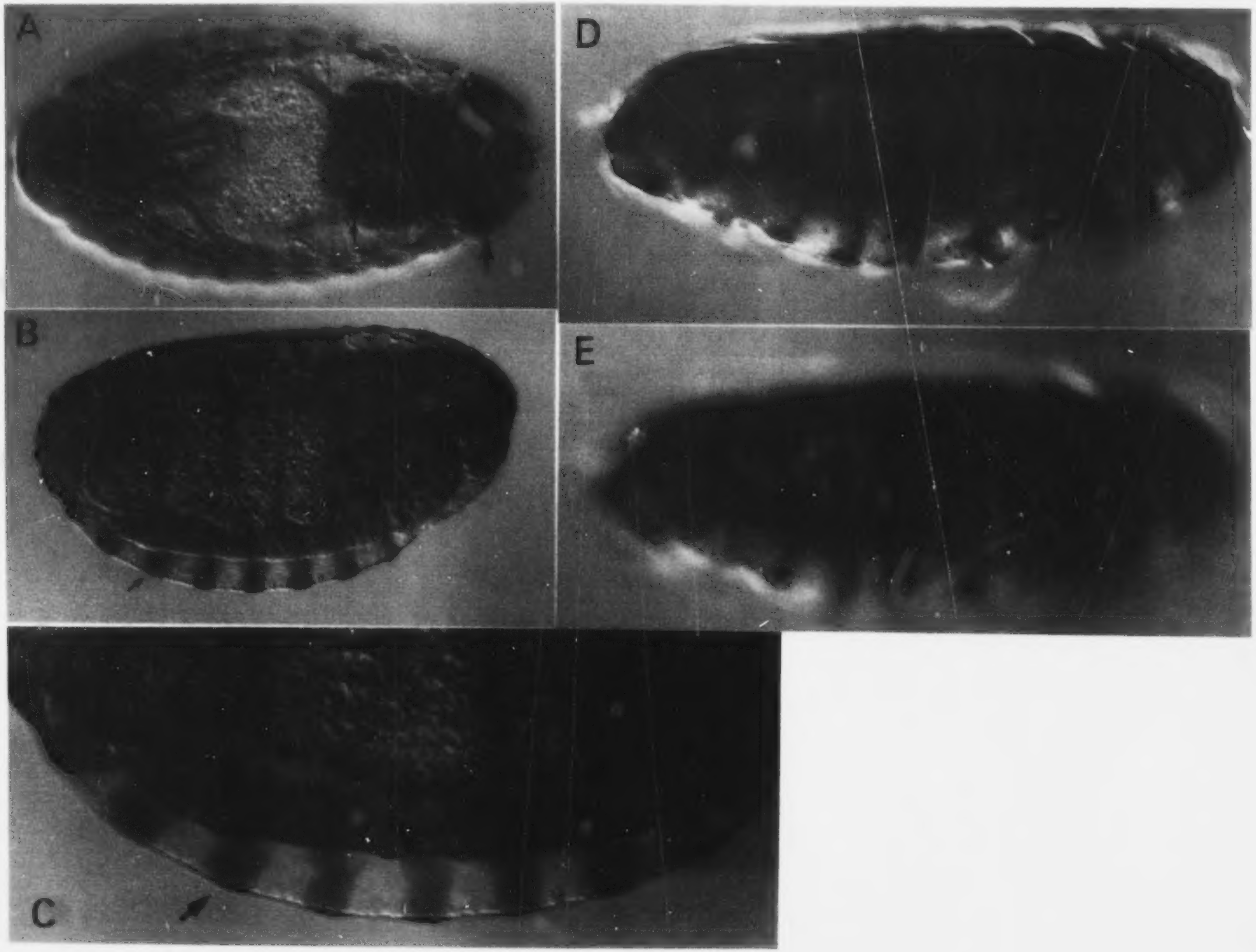
IV.3.2 Developmental expression of the H-39 enhancer-trap and the adjacent transcription unit.

In order to determine if the H-39 flanking genomic DNA contained a transcript which corresponded to the expression pattern of the H-39 insertion, Dr. W.R. Addison probed developmental northern blots with probes derived from p25RL. A 1.6 kB *Hind* III fragment was the only fragment in the region which was found to hybridize to poly (A)+ RNAs from imaginal discs (W. Addison, pers. comm.).The expression of the transcript adjacent to the H-39 insertion and the reporter gene expression were compared to ascertain if they were regulated by the same enhancer elements.

The *lacZ* expression in H-39 is apparent at several stages of development as well as in the regeneration blastema. The reporter gene is expressed in the follicle cells of late ovaries (stage 14 of King (1970)) and during embryogenesis, the *lacZ* pattern is first detected as a faint generalised expression during germ band extension (stages 9 and 10 of Campos-Ortega and Hartenstein (1985). See Figure 21 for examples of H-39 embryonic expression). The expression becomes more intense in the cephalic region of the embryo and in the presumptive anal plate in stage 11. By stages 13 and 14 prominent staining is observed in elements of the peripheral nervous system, a cluster of cells in each optic lobe, and in the ectoderm of the maxillary segment (see Figure 21 A). At stage 15, a striking segmental pattern of expression is observed in what appears to be the hypoderm which will eventually give rise to the ventral denticles belts (see Figure 21 B, C). Expression is also seen in similar stripes in the dorsal hypoderm.

H-39 is expressed along the anterior-posterior boundary and weakly in the posterior compartment of all thoracic and eye-antennal imaginal discs. This is most clearly seen in the wing imaginal discs (see Figure 11 A). The pattern in the leg discs is somewhat different, as the expression in the posterior compartment is stronger relative to the boundary expression, and the boundary expression extends out from from the boundary along the concentric folds of each segment and can be seen to form concentric circles in the leg segments of some leg discs. Double labelling of H-39 wing imaginal discs with antibodies to β-galactosidase and to the *engrailed* protein shows that the expression of H-39 either abuts or only slightly overlaps the expression of *engrailed*. β-galactosidase staining of wing discs from larvae carrying both the H-39 and the E-32 enhancer-trap insertions show only a single line of expression along the a/p boundary, indicating that the expression of the H-39 insertion is contained within or abuts the domain of expression of the enhancer-trap reporting *dpp*. The expression of other *dpp* reporters is known to abut and not overlap the expression of *engrailed* (Raftery et al., 1991).

Figure 21: Embryonic expression of the H-39 insertion and adjacent transcript. A: Head involution stage H-39 embryo with complex expression immunostained with anti-β-galactosidase. Expression in the optic lobe (small arrow) and in the maxillary segment (large arrow) are indicated. B: Late stage H-39 embryo immunostained with anti-b-galactosidase. Segmental ectodermal expression possibly in denticle-secreting cells (arrow). C: Higher magnification of embryo in panel B. Note the thickness of staining cells (arrow) relative to adjacent non-staining cells. D and E: Two focal planes of a late stage Canton-S embryo probed with Dig-labelled RNA complementary to the trancription unit adjacent to H-39 insertion. Note the similarity in hybridization pattern to the lacZ expression pattern in panels B and C.


A
B
C
D
E

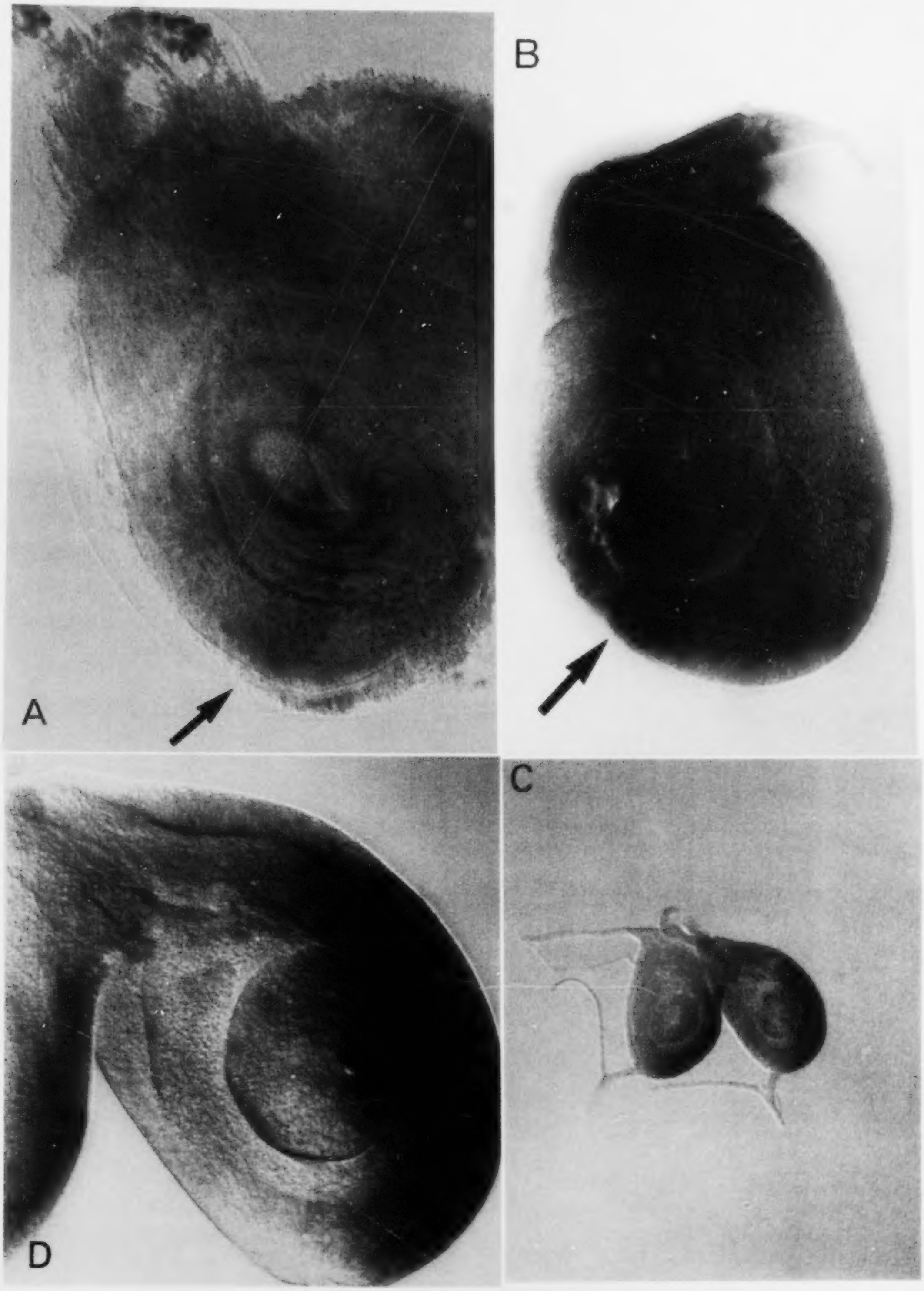
In situ hybridization of RNAs in embryos and imaginal discs was done using digoxigen-labelled RNA probes which were synthesized *in vitro* according to the protocol described in the Methods. The 1.6 kb *Hind* III fragment of p25RL (shown in Figure 19 A) was subcloned into the *Hind* III site of the plasmid Bluescript (SK-). The plasmid was digested with *Eco*R I and used as a template for the T3 RNA polymerase. *In situ* hybridization of this probe to embryos revealed a transcript expressed in a pattern apparently identical to that observed for the β-galactosidase protein in the H-39 insertion line. Especially evident was the late expression pattern in the embryonic hypoderm (Figure 21 D, E). In imaginal discs, the greatest similarity in the patterns of expression was observed in the leg discs. Examples of expression with compartmental (Figure 22 C, D) and compartment boundary expression (Figure 22 B)were observed. Thus the expression in leg discs was clearly the same as the expression of the reporter gene. No consistent pattern of expression was observed in the wing imaginal disc. Some wing discs had elements of the expression pattern seen in the enhancer-trap strain, such as the ventral portion of the a/p boundary. The difference in the success of hybridization may be due to the probe itself. Hybridization of the labelled RNAs made from the 1.6 kb fragment to imaginal disc poly (A)$^+$ RNAs detects several message sizes, perhaps indicating differential processing of the message. It is possible, though unproven, that the probe used may preferentially detect a leg disc specific message. Alternatively, the wing disc expression pattern reported by H-39 may not reflect the expression pattern of the adjacent transcription unit.

The *in situ* hybridization results using a probe from the transcription unit adjacent to H-39 suggests that this is the transcript reported by the insertion. The *in situ* hybridization signal obtained with the probe derived from the 1.6 kb genomic fragment was far less intense than with cDNA probes for the genes *patched*, *dpp* and LF06 (not shown). Strong signals were obtained when using these these probes after only a 20 minute color reaction. With the H-39 genomic probe, the signal was only apparent after 4 hours and the results were much less consistent from disc to disc. This may be due to a low transcript level or possibly because only a small part of the genomic fragment is transcribed. Thus I have shown clearly that the transcript adjacent to the insertion and the reporter in the enhancer-trap are expressed in a very similar pattern, but understanding the significance of the differences in expression in the wing discs must await the isolation of a cDNA probe.

IV.3.3 Cloning of genomic sequences the G-45 insertion

The genomic DNA flanking the G-45[PZ] insertion was also cloned by plasmid rescue. Genomic DNA from the G-45 strain was digested with the enzyme Xba I, circularized with T4 ligase and used to transform *E. coli* DH5α cells to kanamycin resistance. Several dozen colonies were obtained. Plasmid DNA was extracted from a liquid culture from eight of the transformants. Restriction digests of the plasmid DNA derived from all eight colonies was identical. The rescued plasmid contained 2.3 kB of flanking DNA.

Figure 22: Imaginal disc expression of the H-39 insertion and adjacent transcription unit. All discs are oriented anterior left, posterior right. A: *lacZ* expression in H-39 leg imaginal disc detected by immuno staining for anti-β-galactosidase. Arrow indicates the ventral a/p boundary staining. B: Canton-S imaginal leg disc probed with Dig-labelled RNA complementary to the trancription unit adjacent to H-39. Arrow indicates the ventral a/p boundary staining. C: Canton-S leg disc pair probed with digoxigenein-labelled RNA complementary to the trancription unit adjacent to H-39. Expression is restricted to the posterior half of the discs. D: Higher magnification of one of the discs in panel C.


B
A
C
D

The 2.3 kB insert from pG-45-R was excised from the plasmid with *Hind* III, gel purified and used as a probe for *in situ* hybridization to polytene chromosomes. It hybridized to band 100E, the polytene region previously identified with a Carnegie-20 probe as the site of the G-45 insertion, indicating that pG-45-R had sequences from the correct region of the genome (see Figure 23). A deficiency, *Df(3R)faf*^{bp} is deficient for 100E (Fischer-Vize et al 1992). Hybridization of the flanking DNA from pG-45-R to polytene chromosomes from *Df(3R)faf*^{bp}/+ larvae indicate that G-45 insertion is uncovered by the deficiency (see Figure 23).

This has also been confirmed by Southern analysis (see Figure 24). Comparison of hybridization of the flanking probe to genomic DNA digested with *Xba* I and *Eco*R I from G-45 homozygotes and the pre-insertion chromosome reveal a size polymorphism caused by the insertion. As well, comparisons of the *Xba* I and *Eco*R I digests of G-45 / *TM6B*, G-45 / *Df(3R)faf*^{bp}, and *Df(3R)faf*^{bp}/ G-45, indicate that there is a size polymorphism associated with the G-45 and *TM6B* chromosomes, but not with the *Df(3R)faf*^{bp} chromosome. This confirms the *in situ* hybridization result showing that the flanking probe is deleted in the deficiency (Figure 23 C). There is also a faint background banding pattern seen in the southern hybridization (Figure 24) which suggests that the flanking DNA contains a small amount of repetitive sequence.

In situ hybridization to imaginal discs using probes derived from the G-45 flanking DNA are presented in the next chapter.

IV.3.4 Mutagenesis of H-39

Flies homozygous for the insertion H-39 (75DE) are homozygous viable and fertile. A deficiency uncovering the H-39 insertion point, *Df(3L)Cat*^{DH104} (75B8-11;75F1), has been identified previously (MacKay and Bewley 1989). H-39 / *Df(3L)Cat* flies survived as fully fertile adults. Several screens were initiated to identify any excisions of the H-39 element displaying a mutant phenotype when heterozygous with the *Df(3L)Cat* chromosome. The first of these is shown in Figure 25 A. Briefly, this screen involved selecting for loss of the *rosy*$^{+}$ marker in the H-39 insertion chromosome, stocking the excision chromosomes, and scoring for lethality or a visible phenotypes in the homozygotes. Of seventy-nine independent *rosy*$^{-}$ excision chromosomes, 17 were lost and 12 of the remaining 62 were homozygous lethal. No homozygous viable visible phenotypes were observed. Only two of the lethals, Δ26 and Δ54, were also found to be lethal over the Df(3L)Cat deficiency. The other 10 lethals were probably extraneous mutations at second sites caused by the remobilization of H-39, and were discarded.

From the same crossing scheme in Figure 25 A, sixty-nine independent H-39 chromosomes, each from a single male, which retained the *rosy*$^{+}$ marker following a single generation in the Δ2-3 background, were also stocked. None of these chromosomes displayed a lethal or visible phenotype when

Figure 23: *In situ* hybridization of G-45 and Canton-S and *Df(3R) fafbp* chromosomes. A: Hybridization of Carnegie 20 plasmid to G-45 chromosomes. Ectopic hybridization to 100E indicated (arrow) and hybridization to *rosy* indicated (triangle) (*in situ* hybridization performed by Dr. S. Tiong). B: Hybridization of the 2.3 kB *Hind* III fragment from the rescued plasmid, pG-45-R to Canton-S chromosomes. Hybridization to 100E indicated (arrow). C: Hybridization of the 2.3 kB *Hind* III probe to one homologue only in a *Df(3R)fafbp/+* nucleus (arrow).

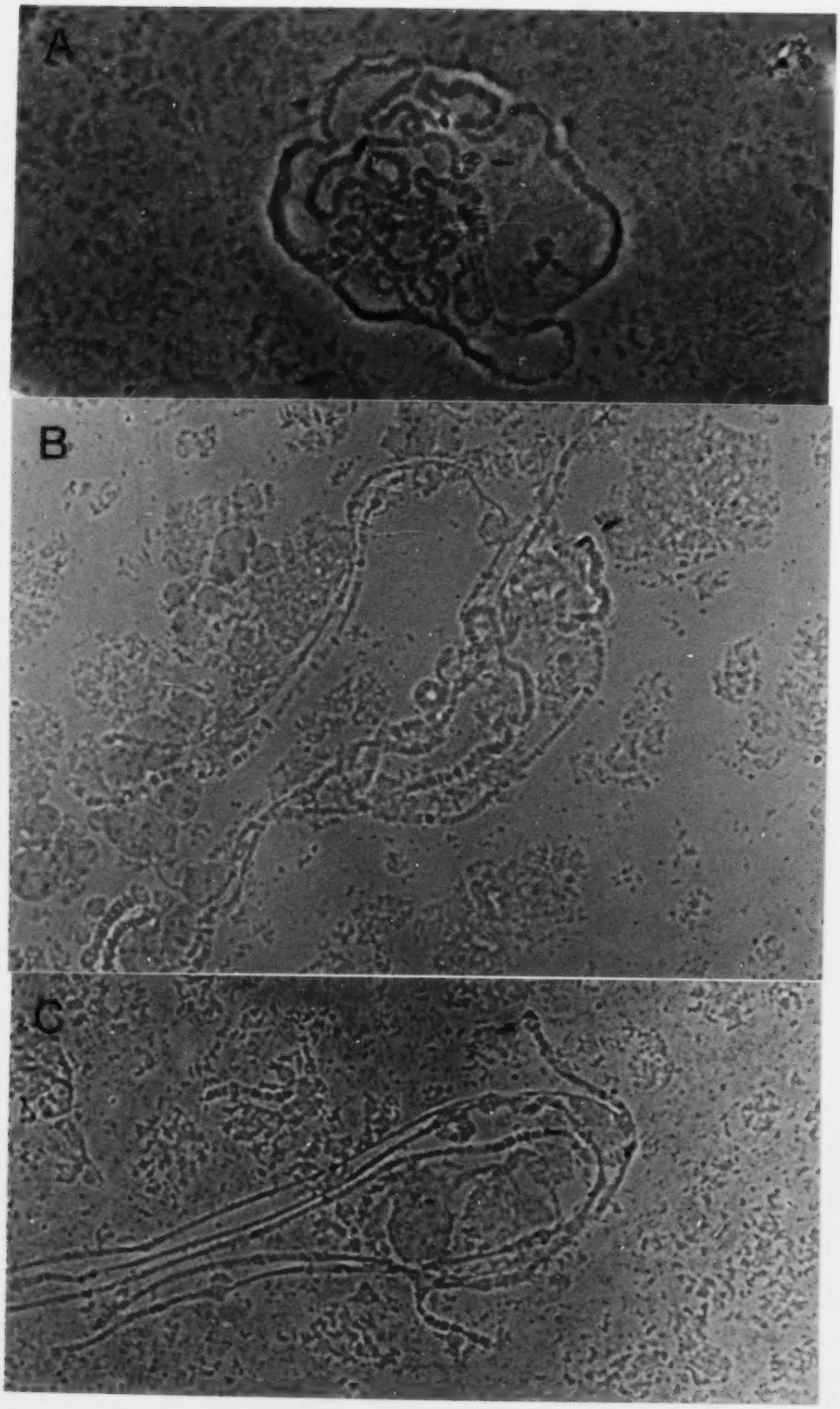
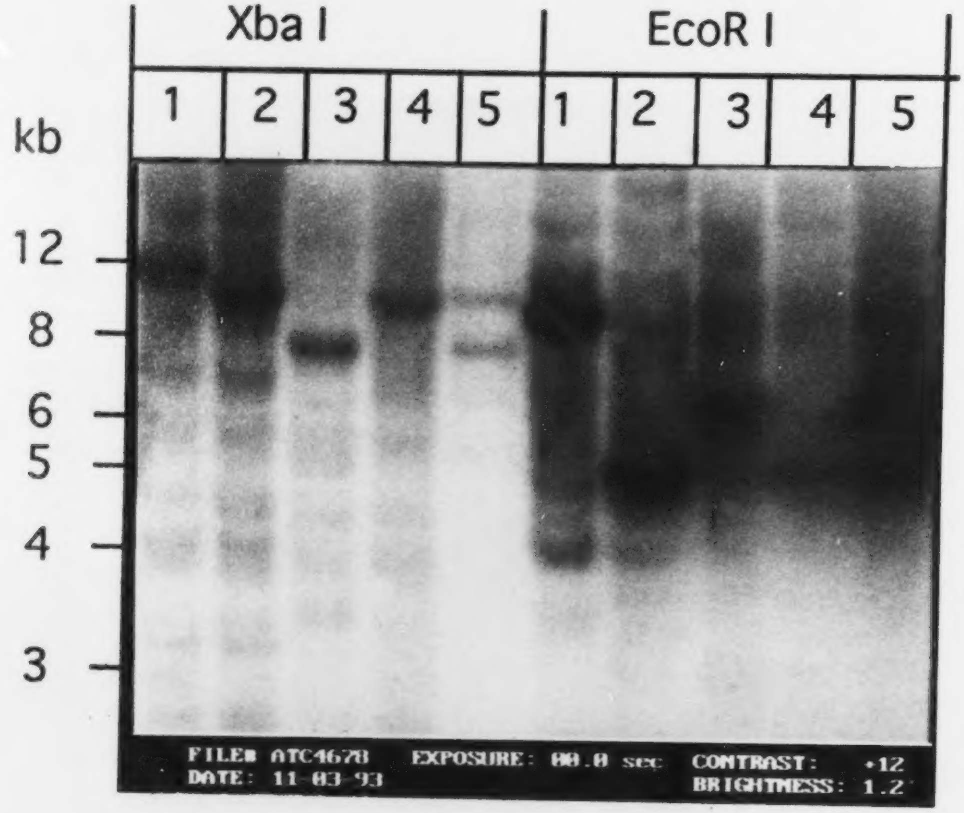

A
B
C

Figure 24: Southern blot hybidization of G-45 DNAs probed with the 2.3 kB *Hind* III fragment from pG-45-R. Genotype 1 represents the pre-insertion chromosome. A size polymorphism is apparent in the insertion strain (compare major signals in lanes 1 and 2: *Xba* I lane 1 has a ~12kB band, lane 2 has a ~10 kB band; *Eco*R1 lane 1 has a ~9 kB band and lane 2 has a ~5 kB band. Both the Xba I and *Eco*R I digests, size polymorphisms are detected for each of the PZ(G-45) and the TM6 chromosomes, however, none is seen for the deficiency (compare lanes 3, 4, 5). This indicates that the probe is deleted from the deletion *Df(3R)fat*$^{bp.}$ G-45 polymorphisms: 10 kb *Xba* I fragment and 5 kB *Eco*R I fragment. TM6 polymorphisms: 8 kb *Xba* I fragment and 6 kB *Eco*R I fragment. In each lane there is a background banding pattern indicating that the probe contains some repetitive sequences, however, there are major bands of hybridization in each band which presumably corrspond to the *in situ* signal at 100E (see Figure 21).


Xba I
EcoR I
1
2
3
4
5
1
2
3
4
5
kb
12
8
6
5
4
3
FILE# ATC4678
EXPOSURE: 00.0 sec
CONTRAST: +12
DATE: 11-03-93
BRIGHTNESS: 1.2

1: +/+

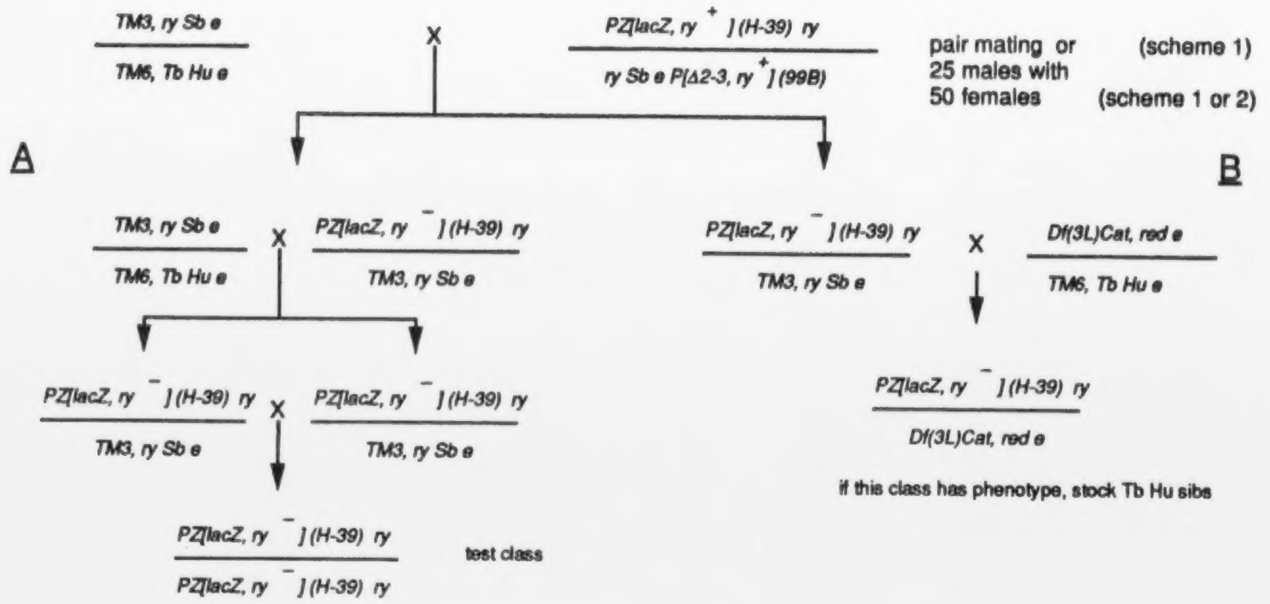
2: PZ(G-45)/PZ(G-45)

3: Df(3R) faf-bp/TM6

4: PZ(G-45)/Df(3R) faf-bp

5: PZ(G-45)/TM6

Figure 25: Excision mutagenesis of H-39. Figure follows on two pages and shows the results of the three schemes employed for generating H-39 excisions (A, B, C). D: Comparison of excision mutagenesis performed in *trans* to an intact or a deleted homologue.


TM3, ry Sb e / TM6, Tb Hu e
X
PZ[lacZ, ry+](H-39) ry / ry Sb e P[Δ2-3, ry+](99B)
pair mating or (scheme 1)
25 males with
50 females (scheme 1 or 2)
A
TM3, ry Sb e / TM6, Tb Hu e
X
PZ[lacZ, ry−](H-39) ry / TM3, ry Sb e
PZ[lacZ, ry−](H-39) ry / TM3, ry Sb e
X
PZ[lacZ, ry−](H-39) ry / TM3, ry Sb e
PZ[lacZ, ry−](H-39) ry / PZ[lacZ, ry−](H-39) ry
test class
B
PZ[lacZ, ry−](H-39) ry / TM3, ry Sb e
X
Df(3L)Cat, red e / TM6, Tb Hu e
PZ[lacZ, ry−](H-39) ry / Df(3L)Cat, red e
if this class has phenotype, stock Tb Hu sibs

H-39 excision scheme 1:

62 independent ry– events
(26 from 566 pair matings, 17
of these lost, 53 from 109
bottle matings)

12 homozygous lethal

2 fail to complement Df(3L) Cat

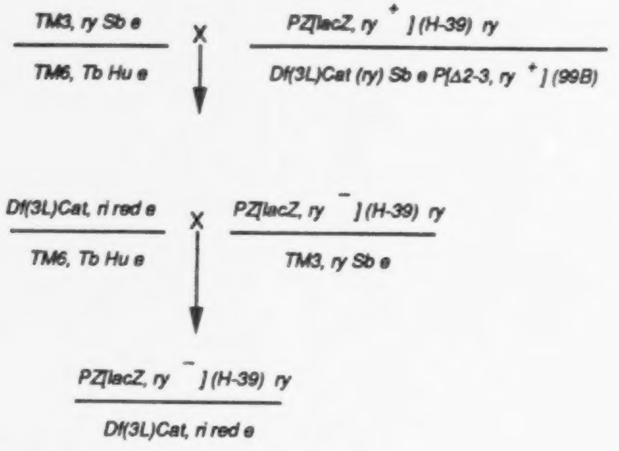
other lethals are presumably
at second sites

H-39 excision scheme 2:

≥69 independent ry– events
(129 events tested from 69
bottles of 177 bottles total)

3 fail to complement Df(3L) Cat

C


TM3, ry Sb e
TM6, Tb Hu e
X
PZ[lacZ, ry +] (H-39) ry
Df(3L)Cat (ry) Sb e P[Δ2-3, ry +] (99B)
Df(3L)Cat, ri red e
TM6, Tb Hu e
X
PZ[lacZ, ry −] (H-39) ry
TM3, ry Sb e
PZ[lacZ, ry −] (H-39) ry
Df(3L)Cat, ri red e

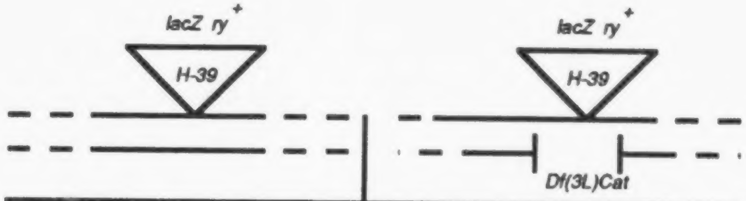
25 males with
50 females

H-39 excision scheme 3:

≥47 independent ry− events
(127 tested from 47 bottles of
71 bottle matings total)

1 lethal failed to complement
Df(3L) Cat, Δ3002

D


lacZ ry +
H-39
lacZ ry +
H-39
Df(3L)Cat

69 independent events in 177 bottles	47 independent events in 71 bottles
129 total events in 177 bottles	137 total events in 71 bottles
3 lethals	1 lethal

heterozygous with the *Df(3L)Cat* chromosome. Inspection of the imaginal disc staining patterns revealed, however, that 12 of the 69 H-39 derivatives had altered imaginal disc *lacZ* expression. Four of the twelve had no imaginal disc expression. Such striking and frequent changes in the staining pattern suggests that there may be frequent mobilization without concomitant loss of the P-element from the genome.

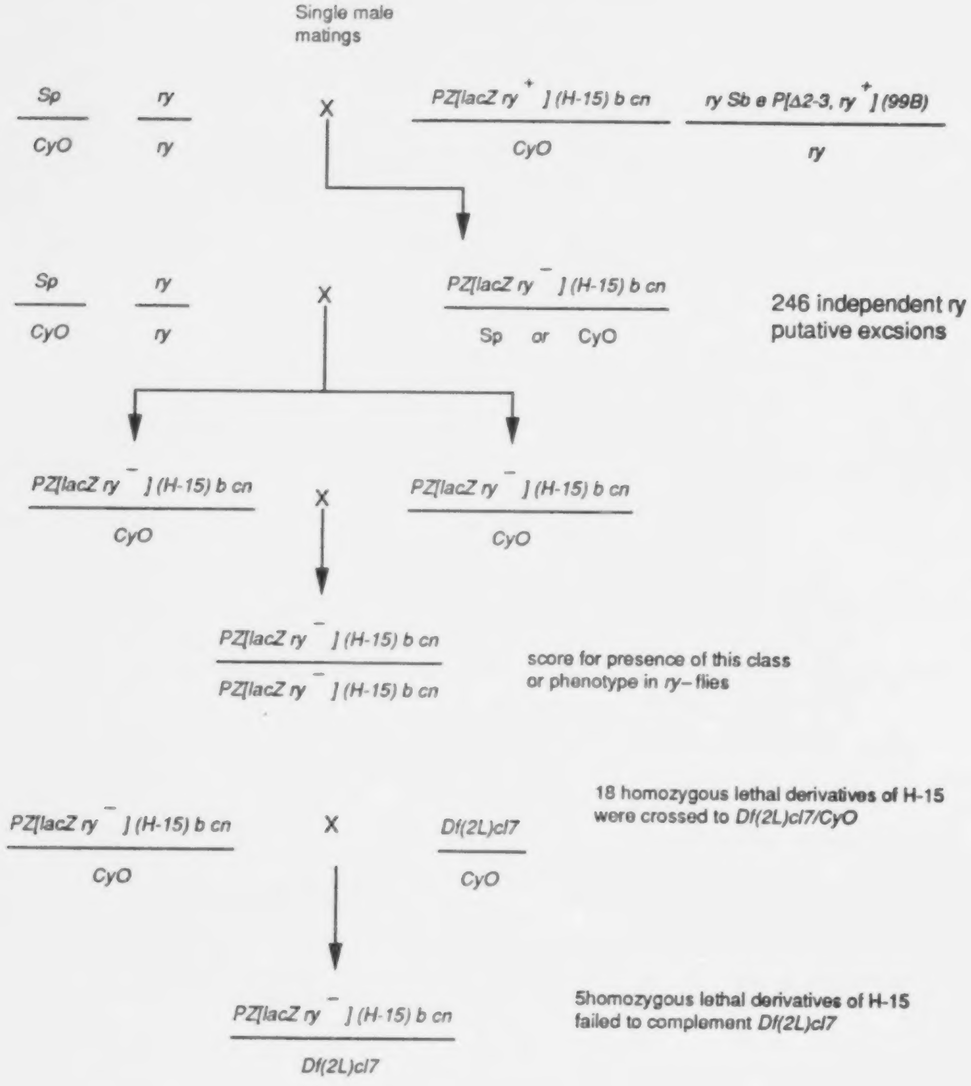
A second scheme in which the excision chromosomes were tested over the *Df(3L)Cat* chromosome, doing away with the stocking generation of the first scheme, is also indicated in Figure 25 B. From this scheme, a total of 129 *rosy⁻* excisions representing at least 69 independent events were isolated. Three of the excisions, Δ32, Δ174 (which was subsequently lost), and Δ215 were found to be lethal over the *Df(3L)Cat* chromosome. Again, no visible viable mutations were observed.

One last variation was attempted for the generation of excision chromosomes. It has been reported that high frequencies of precise P-element excision is dependent on the presence of a wild-type homologue (Engels at al 1990). To see if this could be exploited to generate more efficiently small flanking mutations, H-39[PZ] excisions were generated with the element in *trans* with a deficiency on the other homologue. A chromosome carrying both the *Df(3L)Cat* deficiency and the Δ2-3 element was constructed. The scheme is shown in Figure 25 C. One hundred and thirty seven *rosy⁻* excisions, representing at least 47 independent events were screened and one lethal excision (Δ3002) was isolated. The frequency of lethals per independent excision was apparently lower than in the first approach (1/47 , 0.02 versus 3/69 , 0.04, see Figure 25 D). However, only one event was observed. In any case, the results of the two approaches were not very different.

IV.3.5 Mutagenesis of H-15

The H-15 strain was determined to have PZ insertions at 25F and 58CD. The imaginal disc staining pattern in the ventral leg and antennal discs was found to be linked to the marker *Sternopleural* (Sp) and not to the marker *Pin-yellow-tip* (Pin^{yt}) indicating that the insertion of interest was at 25F. The insertions were separated from each other by recombination, resulting in a PZ(H-15, 25F), b cn chromosome. The 25F insertion was found to be semi-lethal over the interval 25F1-2 based on semilethality over Df(2L)Gdh-α (5% of expected progeny), Df(2L)cl1 (10%), Df(2L)cl7 (8%), Df(2L)clh2 (7%) and viable over the deficiencies Df(2L)clh4(85%), Df(2L)clh1(25%) and Df(2L)E66 (65%) (references for the deficiencies: Szydonia and Reuter. 1988; Tartof et al., 1989). The semi-lethality was caused by the insertion as two of three homozygous viable *rosy⁻* excisions of the insert (chosen at random from the excision mutagenesis scheme described in Figure 26) were shown to be completely viable over a deficiency for the 25F2-5 interval. The third was still hemizygous semi-lethal and was presumed to be an imprecise excision.

Figure 26: Generation of excisions from the insertion H-15.


Single male matings
Sp / CyO
ry / ry
X
PZ[lacZ ry+] (H-15) b cn / CyO
ry Sb e P[Δ2-3, ry+] (99B) / ry
Sp / CyO
ry / ry
X
PZ[lacZ ry−] (H-15) b cn / Sp or CyO
PZ[lacZ ry−] (H-15) b cn / CyO
X
PZ[lacZ ry−] (H-15) b cn / CyO
PZ[lacZ ry−] (H-15) b cn / PZ[lacZ ry−] (H-15) b cn
PZ[lacZ ry−] (H-15) b cn / CyO
X
Df(2L)cl7 / CyO
PZ[lacZ ry−] (H-15) b cn / Df(2L)cl7

246 independent ry putative excsions

score for presence of this class
or phenotype in *ry*− flies

18 homozygous lethal derivatives of H-15
were crossed to *Df(2L)cl7/CyO*

5homozygous lethal derivatives of H-15
failed to complement *Df(2L)cl7*

18 of 246 independent *rosy*$^-$ excisions of the H-15 insertion were found to be homozygous lethal (Figure 26). Five of the eighteen excisions (e142, e200, lethal 11, lethal 14, and lethal 17) were found to be lethal when heterozygous with Df(2L)cl7. The other thirteen were presumably extraneous second site lethals and were not studied further. The five lethal excisions were found to complement *midline* (mid) an embryonic lethal mutation in 25F1-2 but failed to complement Df(2L)E66, a deficiency which complemented the haplo-dependent semi-lethality of the H-15 insertion. This suggests that the excisions extend beyond the complementation group disrupted by the H-15 insertion. It has been confirmed that at least three of the five excisions (e142, e200, and lethal 11) are cytologically visible deficiencies, e142 has been designated a deficiency for 25F2-3 to 26A1 (D. Long, pers. comm.).

IV.3.6 Mutagenesis of G-45

G-45 is a homozygous viable insertion in band 100E. The G-45 excision mutagenesis is shown in Figure 27. Sixty of 252 *rosy*$^-$ excisions were found to be homozygous lethal. Forty-four of the excisions were found to be lethal in *trans* to a deficiency for 100E, Df(3R)faf^{bp}.

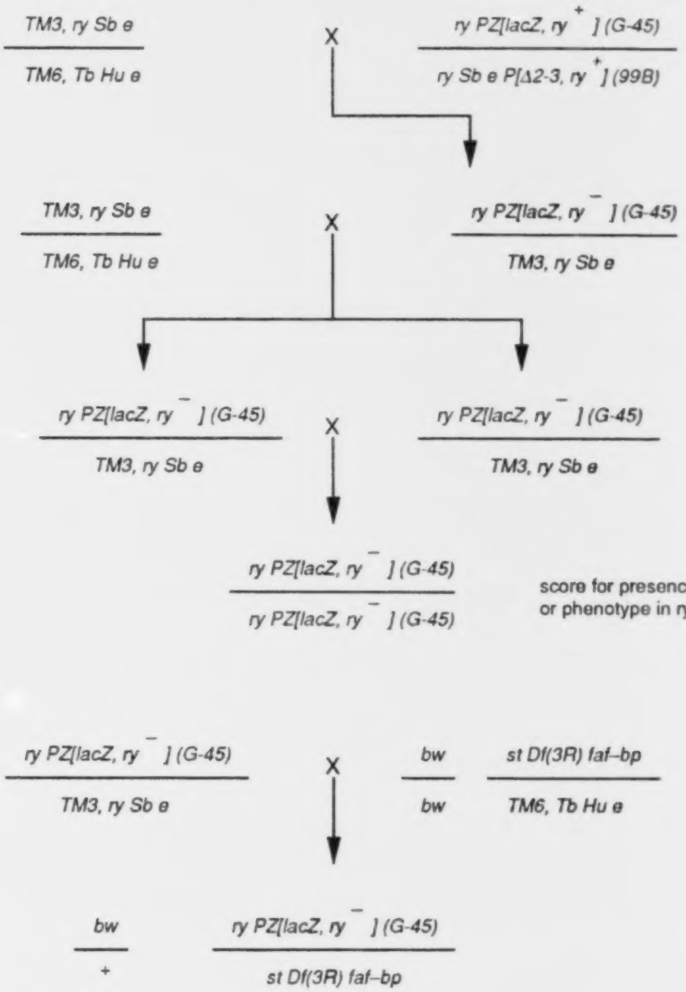
IV.3.7 Comparison of mutagenesis of G-45, H-15, and H-39

Table 9 summarizes the mutagenesis of G-45, H-15 and H-39. Some substantial differences were observed between the three loci. Although precise rates cannot be calculated from the data, the observed frequencies of excision were very different . The majority of the H-39 crosses were set up in bottles with 25 PZ / Δ2-3 males and 50 balancer females. On average less than one excision was recovered per bottle. G-45 PZ / Δ2-3 and H-15 PZ / Δ2-3 males were mated individually with 3-5 balancer females and virtually all G-45 vials and roughly half of the H-15 vials yielded at least one excision. This suggests that there may be as much as a 25-fold difference in the excision rate between G-45 and H-39. The rates of lethal excisions produced per excision varied greatly as well. H-15 and H-39 were quite similar with lethals per excision in the range of 2-3%, while G-45 yielded 17 % lethal excisions. In view of its location close to the tip of 3R, the high rate of lethals in G-45 excisions may be explained by the production of terminal deficiencies during P-element excision which has been described by Tower et al. (1993).

IV.4 Genetics of H-39 excision mutants.

The excision mutants Δ26, Δ32, Δ54, Δ215, and Δ3002 were mapped by Southern analysis and all five contained rearrangements adjacent to the H-39 insertion (W. Addison pers. comm., see Figure 28). The excisions, Δ215 and Δ3002 are deleted for the entire cloned region, while Δ26, Δ54, and Δ3002 all contain rearrangements on the left side of the insertion. Only Δ215 and Δ3002 are definitively deleted in the transcription unit on the right side reported by H-39 *lacZ* expression. The present results do not preclude the possibility that the

Figure 27: Generation of excisions from the insertion G-45.


TM3, ry Sb e / TM6, Tb Hu e
X
ry PZ[lacZ, ry+] (G-45) / ry Sb e P[Δ2-3, ry+] (99B)
TM3, ry Sb e / TM6, Tb Hu e
X
ry PZ[lacZ, ry−] (G-45) / TM3, ry Sb e
ry PZ[lacZ, ry−] (G-45) / TM3, ry Sb e
X
ry PZ[lacZ, ry−] (G-45) / TM3, ry Sb e
ry PZ[lacZ, ry−] (G-45) / ry PZ[lacZ, ry−] (G-45)
ry PZ[lacZ, ry−] (G-45) / TM3, ry Sb e
X
bw / bw
st Df(3R) faf–bp / TM6, Tb Hu e
bw / +
ry PZ[lacZ, ry−] (G-45) / st Df(3R) faf–bp

single male matings

252 independent ry- excisions

score for presence of this class or phenotype in ry– flies

60 homozygous lethal derviatives were found and crossed to Df(3R)faf-bp

44 homozygous lethal derviatives failed to complement Df(3R)faf-bp

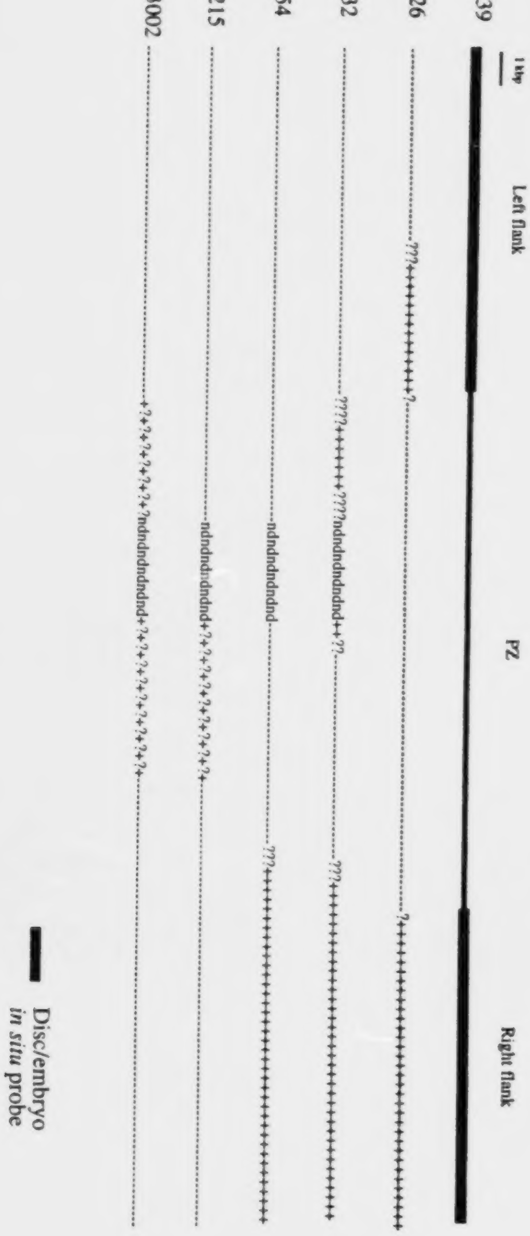
Table 9: Comparison of excision mutagenesis of G-45, H-15 and H-39.

insertion	excision rate[1]	lethals/excision	lethals at locus[2]/excision
G-45	high	0.24	0.17
H-15	high	0.07	0.02
H-39	low	0.19[3]	0.04

1. See section IV.3.7 for details
2. Lethal excisions which fail to complement a deficiency which uncovers the insertion point.
3. Caculated based on 12 homozygous lethal excisions from scheme 1 (see Figure 25 A).

Figure 28: Map of rearrangements caused by H-39 excisions. Figure courtesy of Dr. W.R. Addison.

-----------	sequence deleted
++++++	sequence present
??????	rearrangement endpoint uncertain
?+?+?+	sequence present, but genomic location uncertain
ndndnd	not tested


H39
Δ26
Δ32
Δ54
Δ215
Δ3002
1 kb
Left flank
PZ
Right flank
Disc/embryo in situ probe

other insertions delete regulatory or as yet unmapped regions of the H-39 transcription unit.

The five excisions all fail to complement one another, showing that they all delete at least one common vital gene. Further complementation was carried out between the excision mutations and several preexisting deficiencies and mutations in the region and the results are summarized in Figure 29. All five H-39 excisions fail to complement the *Df(3L)Cat* deficiency (75B8-11; 75F1) and complement the deficiencies *Df(3L)W*^{r4} (75B8-11; 75C5-7) and *Df(3L)W*^{r10} (75A6-7; 75C5-7). Three previously uncharacterized mutants were provided by Dr. Kristen White. These were derived as X-ray induced w^- revertants of a P[w^+] element which was inserted in 75C. The three mutants, X22, X31 and X33 were lethal over *Df(3L)Cat* and only X22 was lethal over *Df(3L)W*^{r4}. Δ26, Δ215, and Δ3002 all complemented X22, X31 and X33, Δ54 failed to complement X33, and Δ32 failed to complement all three of the X-ray induced mutations.

It is possible to construct a genetic complementation map of the region from this data. The mutants define at least three complementation groups in the region uncovered by Df(3L)Cat but not Df(3L)W^{r4} (see Figure 29). Δ26, Δ215, and Δ3002 behave as though they delete just one of the three intervals. Δ32 is deleted for all three of the intervals and Δ54 for two of them. The limitation of this analysis is that the number of complementation groups present in the common interval uncovered by Δ26, Δ215, and Δ3002 is still unknown. It is therefore premature to equate the lethal complementation group shared by all five excisions with the transcription unit reported by H-39.

The lethal stage of the excisions was also considered. All mutants were balanced over TM6B which carries the dominant marker *Tubby* (*Tb*) which can be scored reliably from late second instar onwards. No Tb^+ larvae or pupae were observed in the stocks of the excisions implying that the chromosomes were homozygous embryonic or early larval lethal. Given the possibilty of second site lethals generated during the excision mutagenesis, all possible transheterozygous combinations of the excsions Δ26, Δ54, Δ215, and Δ3002 tested for lethal stage. Δ32 was excluded from the analysis as it is apparently deleted for several vital functions. Also, to avoid the potential ambiguity of scoring second instar larvae for the marker *Tb* , I made use of the *lacZ* expression in H-39 as a dominant larval marker. Each of the four excisions was crossed to the H-39 enhancer-trap line and all six pairwise combinations of H-39/ΔH-39$_x$ XH-39/ΔH-39$_y$ were set up in both directions The results are shown in Table 10. Crosses between H-39/Δ26 and H-39/Δ54 resulted in 27% embryonic lethality implying that they are deficient for a common early essential function. The lethal embryos had a normal cuticular pattern. For all other combinations, only a very low frequency of embryonic lethality was observed ($\leq$ 6%) and the lethal embryos displayed no obvious cuticular abnormalities. Survivorship during larval stages was estimated by selecting a sample of 48-72h, 72-96h, or 96-120h old larvae and assaying them for H-39 *lacZ*

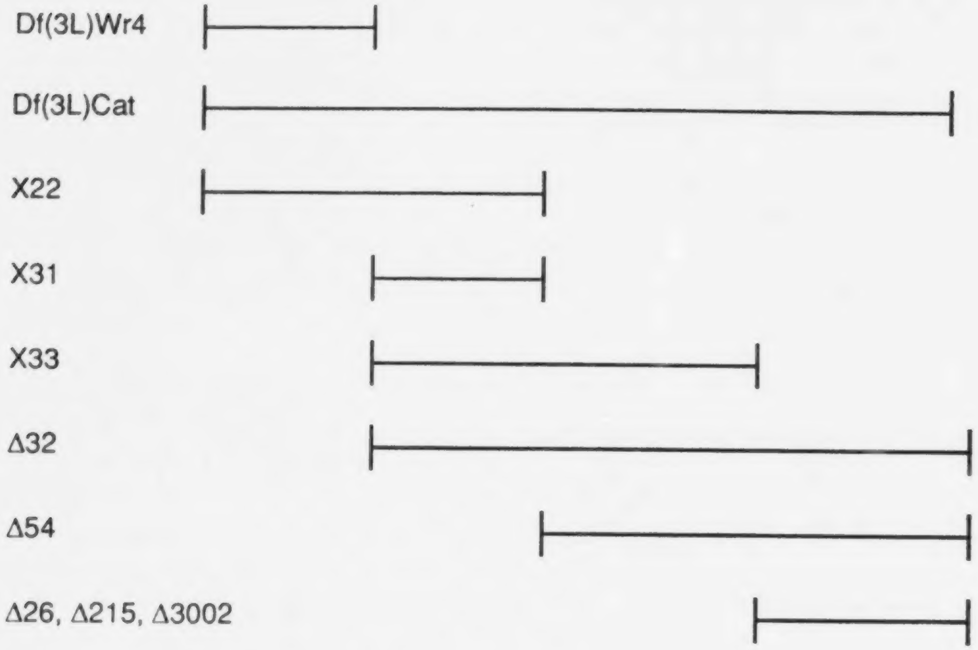

Df(3L)Wr4
Df(3L)Cat
X22
X31
X33
Δ32
Δ54
Δ26, Δ215, Δ3002

Figure 29: Lethal complementation map of H-39 excisions and other deficiencies in the 75CD region.Failure of two mutations to complement is denoted by overlapping black lines.

Table 10 Lethal stages of transheterozygous H-39 excisions in crosses of H-39/H-39Δ_x X H-39/H-39Δ_y.

H-39/H-39Δ_x X H-39/H-39Δ_y	% dead embryos	($\Delta x/\Delta y$ larvae surviving)/ (total larvae) sampled at timepoint:			(escaper adults/ total adults eclosing)
		48-72 hours	72-96 hours	96-120 hours	
26 x54	27 (50/185)	1/57	2/64	1/102	0/296
26 x 215	3 (5/183)	nd	nd	16/124	8/286
26 x 3002	5 (7/148)	nd	nd	28/159	0/355
54 x 215	4 (12/306)	nd	nd	12/101	6/235
54 x 3002	2 (6/266)	nd	nd	18/103	0/212
215 x 3002	6(16/306)	1/58	1/44	0/107	0/211

Crosses were set up in both directions and the data was pooled as no differences were seen in the frequencies. Larvae with no H-39 lacZ staining pattern were scored as $\Delta x/\Delta y$ larvae.

expression. Analysis of late third instar larvae showed that crosses between Δ26 or Δ54, and Δ215 or Δ3002 resulted in moderate survival of the deficiency *trans*-heterozygotes to third instar. Third instar survivors comprised 12 to 18 % of the sample, compared to the expected Mendelian total of 25%. Crosses between Δ26 and Δ54, or Δ215 and Δ3002 resulted in significantly lower survival of second and third instar *trans*-heterozygous larvae. In all crosses, surviving *trans*-heterozygous third instar larvae (72-96 hour and 96-120 hour samples) had apparently normal imaginal discs. The low proportion of Δ26/Δ54 and Δ215/Δ3002 surviving to second instar is consistent with the embryonic lethality of the Δ26 by Δ54 cross and implies the lethal stage of the Δ215 by Δ3002 cross is predominantly the first larval instar. No adult escapers (identified as ry eyed flies) were observed in crosses between Δ26, Δ54, and Δ3002, or between Δ215 and Δ3002. Escapers were observed in crosses between Δ26 or Δ54, and Δ215 at a rate of up to 10 % of the expected number. The escapers seemed morphologically normal but weak and died within two days of eclosion.

These results are consistent with all four excisions having a common pupal lethal mutation and as well as an embryonic lethal shared by Δ26 and Δ54 and a further early larval lethal shared by Δ215 and Δ3002. Alternatively, the lethality may be due to the same complementation group and the two pairs of excisions could partially complement each other. The first explanation is simpler and is also consistent with the molecular characterization of the excisions in that Δ26 and Δ54 are both rearranged in the left flank of H-39 while Δ215 and Δ3002 share a deletion of the right flank. However, it is still impossible to tell which if any of the lethals is due to disruption of the transcription unit reported by H-39.

IV.5. Chapter Summary.

In this chapter the characterization of several of the enhancer traps expressed in regenerating imaginal discs was presented. Three of the insertions, B-82, E-32 and AD-55 were found to correspond to the previously described loci *crb*, *dpp* and LF-06. The preliminary genetic charcterization of four other insertions, C-92, G-45, H-15, and H-39 was also performed, and genomic DNA flanking the insertions G-45 and H-39 was cloned. There was also evidence that the genomic DNA flanking the insertion H-39 contained a transcript expressed in the same pattern as the H-39 enhancer-trap. This data is summarized in Table 11.

Table 11: Summary of data in chapter IV.

Insertion/Class	cytological location	Notes
B-82 (I)	95F	allelic to *crumbs*, insertion causes mutation (see Figure 15)
C-92 (II)	42A	localized to 42A2-10, insertion causes lethality.
E-32 (II)	22F	Inserted in *transcript-near-decapentaplegic* (see Figure 17). The insertion reports dpp and tnd expression.
F-45 (II)	91BC	Homozygous lethal. Not characterized.
G-45 (II)	100E	Homozygous viable insertion. Flanking DNA cloned by plasmid rescue (see Figures 23 and 24). Lethals at or near locus generated by P-element excsions (Figure 27).
H-09 (V)	72EF	Homozygous viable insertion. Expression pattern similar to wingless, however, not inserted at the wingless locus (28A).
H-15 (II)	25F, 58CD	Homozygous viable (but semi lethal for deficiencies of 25F2-5, semi-lethality is caused by insertion, see section IV.3.5). Lethal excisions thus far characterized are cytologically visible deficiencies
H-21 (I)	50AB	Homozygous viable. Not characterized.
H-39 (II)	75DE	Homozygous viable insertion. Flanking genomic DNA cloned by plasmid rescue and subsequent screening of genomic DNA library with rescued flank (see Figure 19, 20). Transcription unit in right flank is expressed in a pattern similar to the enhancer trap reporter expression (Figures 21 and 22). Two lethal excisions generated at the location, Δ215 and Δ3002 delete the trancription unit (Figure 28) however, they may contain more than one mutation (Figure 29 and Table 10) and it is not yet possible to assign the lethality to the transcription unit reported by H-39.
AD-55 (I)	60CD	Reports the same trancription unit as previously characterized LF-06 enhancer trap. LF-06 cDNA encodes a novel protein.

V. GENE EXPRESSION DURING IMAGINAL DISC REGENERATION

The previous two chapters describe the identification and preliminary characterization of enhancer-traps expressed during imaginal disc regeneration. This final chapter of results describes two groups of experiments. The first involves an analysis of the timing of the expression of the enhancer-traps during disc regeneration, with the goal of understanding how the expression may relate to previously described events during disc regeneration. The expression of the segment polarity genes *wingless* and *engrailed* during regeneration is described to provide molecular markers for events during pattern regulation. The second set of experiments is designed to see whether expression of any of the enhancer-traps in the *su(f)* background corresponds to that of a nearby transcription unit. The insertions tested included E-32, AD-55, and B-82 for which the *dpp*, *tnd*, and LF-06 cDNA clones and monoclonal antibody for the *crumbs* protein were already available and G-45 for which flanking genomic DNA clones were isolated in this study.

V.1 Time course of enhancer-trap and segment polarity gene expression in the *su(f)* cell lethal background.

V.1.1 Time course of enhancer-trap expression in *su(f)*

In order to determine when the expression is first induced in regenerating *su(f)* imaginal discs, I examined discs from the insertion lines at several time points following a 48 h 29°C heat pulse in the *su(f)* background. The enhancer-traps expressed during disc regeneration were originally selected because they were expressed 24 h following a 48 h heat pulse at 29°C. Furthermore, all insertions (except C-39 which was from class III) were known not to be induced by a 24 h heat pulse. The results of a time course of enhancer-trap expression are summarized in Table 12. All but two insertions were found to be expressed as early as the end of the heat pulse. B-17 and F-45 were not observed to be expressed until 24 hours following the heat pulse. In general, the intensity and extent of the *lacZ* expression increased throughout the time course. The relative stability of the β-galactosidase protein makes it possible that the apparent increase in expression represents the cumulative effects of transient responses in successive groups of cells. In any case, these results indicate a very early transcriptional response during regeneration.

V.1.2 Time course of segment polarity gene expression in *su(f)*

Due to the unexpectedly uniform early response of the enhancer-traps in the time-course, I examined the expression of the segment polarity genes *wingless* and *engrailed* during regeneration for comparison. The expression of *engrailed* marks the cells of posterior compartment (Raftery et al., 1991; but see also Blair, 1992). The expression of *wingless,* which encodes a secreted protein of the Wnt family (van den Heuvel et al., 1989) is restricted in a single wedge in the ventral region of the anterior compartment and the posterior margin of *wingless* expression abuts but does not overlap the expression domain of the

Table 12: Ectopic *lacZ* expression at various time points following a 48 h heat pulse.

Strain	(- 24 h)	(0 h)	(6 h)	(12)	(18)	(24)
B-17	−	−	−	−	−	+
B-82	−	+	+	+	+	+
C-07	−	+	+	+	+	+
C-39	+	+	+	+	+	+
C-76	−	+	+	+	+	+
C-92	−	+	+	+	+	+
D-42	−	+	+	+	+	+
D-46	−	+	+	+	+	+
E-17	−	+	+	+	+	+
E-32	−	+	+	+	+	+
E-34	−	+	+	+	+	+
E-37	−	+	+	+	+	+
E-60	−	+	+	+	+	+
E-91	−	+	+	+	+	+
F-22	−	+	+	+	+	+
F-36	−	+	+	+	+	+
F-45	−	−	−	−	−	+
G-45	−	+	+	+	+	+
H-15	−	−	+	+	+	+
H-21	−	+	+	+	+	+
H-39	−	+	+	+	+	+
H-44	−	+	+	+	+	+
H-87	−	+	+	+	+	+
AD-55	−	+	+	+	+	+

+ = ectopic expression

− = normal expression

engrailed protein (Couso et al., 1993). Changes in expression of these genes may be useful as molecular markers of early stages of regeneration and duplication events.

The expression of*wingless* expression was monitored with an enhancer-trap element inserted at the *wingless* locus (Perrimon et al., 1991) which has been demonstrated to be expressed in a pattern very similar to that of the *wingless* protein (Williams et al., 1993; Couso et al., 1993). The *wingless* enhancer-trap was crossed into the *su(f)* background and the expression was assessed at several timepoints after a 48 h heat pulse at 29°C. Very little change in *wg-lacZ* expression was observed immediately following the heat pulse (0 h), although in some discs a slight extension from the anterior margin of *wingless* expression was observed. By 6 h after the heat pulse, a very pronounced stretching of *wg-lacZ* expression along the anterior margin towards the dorsal part of the disc was observed in several discs. In three discs, two discrete patches of *lacZ* staining were observed, as would be expected in leg duplications (see Figure 30).

Discs with an extra patch of *wg-lacZ* expression were seen more frequently in discs dissected at 18 and 24 h after the end of the heat-treatment (Table 13). The frequency of ectopic expression observed per leg disc at the 24 h time point (7 %) is somewhat lower than the frequency of 24.5 % reported by Girton and Russell (1980) for duplications per mesothoracic leg. That frequency is based on all duplication events found in mesothoracic legs. It is possible that only some duplication events involve extra patches of *wg* expression or that the trend of increasing frequency of ectopic *wingless* patches continues past 24 hours. The *wg-lacZ* expression was also scored in wing discs. The expression was somewhat irregular in appearance but there were no ectopic patches similar to those seen in the leg discs. This is consistent with the ectopic expression being associated with duplication events as the distribution of *su(f)* induced lesions in the wing disc results in regeneration rather than duplication (Clark and Russell, 1977; Russell et al., 1977).

The expression of *engrailed* and *invected* were detected using a monoclonal antibody supplied by Dr. Pat O'Farrell, UCSF (anti-*en/inv*). No ectopic patches of *engrailed* protein expression were observed even as late as 24 hours following the heat pulse in the *su(f)* background, but the intensity of expression in the peripodial membrane increased in comparison to untreated imaginal discs.

H-15 is a class II insertion with an embryonic expression profile like that of many segment polarity genes and with expression in the ventral portion of the leg and antenna discs (see Figure 18). It was also examined during a time course in the *su(f)* mutant background. H-15 expression was not affected until 6 h after the 48 h heat treatment. The patterns of spreading and ectopic patches seen were similar to those seen for *wingless* expression (see Figure 31). This is interesting because H-15 is expressed ectopically following the induction of ectopic *wingless* expression in the anterior dorsal compartment but not in the posterior dorsal compartment (pers. comm. Dr. B. Wilder Dept. of Genetics,

Figure 30: *wg-lacZ* expression in heat treated *su(f)* leg discs. Discs in panels A-D are oriented dorsal up, ventral down, and anterior (a) and posterior (p) are indicated. All are seen in apical aspect. Discs are immunostained with anti-β-galactosidase except for panel B which is stained with X-gal. A: Control *wg-lacZ* leg disc with characteristic anterior ventral wedge of expression. B: *su(f);wglacZ* disc six hours after a 48 hour heatpulse. Note the *wg-lacZ* expression extending dorsally (arrow). C and D: Similar extension (arrows) in *su(f); wg-lacZ* leg discs 24 h after a 48 heat treatment. Note that in D the expression has extended nearly to the dorsal a/p compartment boundary (compare with *wg* and *en* double labelled disc,Figure 33 A) and that the expression between the ectopic dorsal patch and the original ventral patch is less intense than in the dorsal patch. E-G: Several focal planes of an anterior aspect of a *su(f); wg-lacZ* leg disc 24 h after a 48 h heat-treatment. Apical (ap), basal (ba), dorsal(d), and ventral (v) aspects are indicated. A discrete patch of ectopic *wg-lacZ* expression is indicated (arrows). E through G are progressively deeper planes of focus.

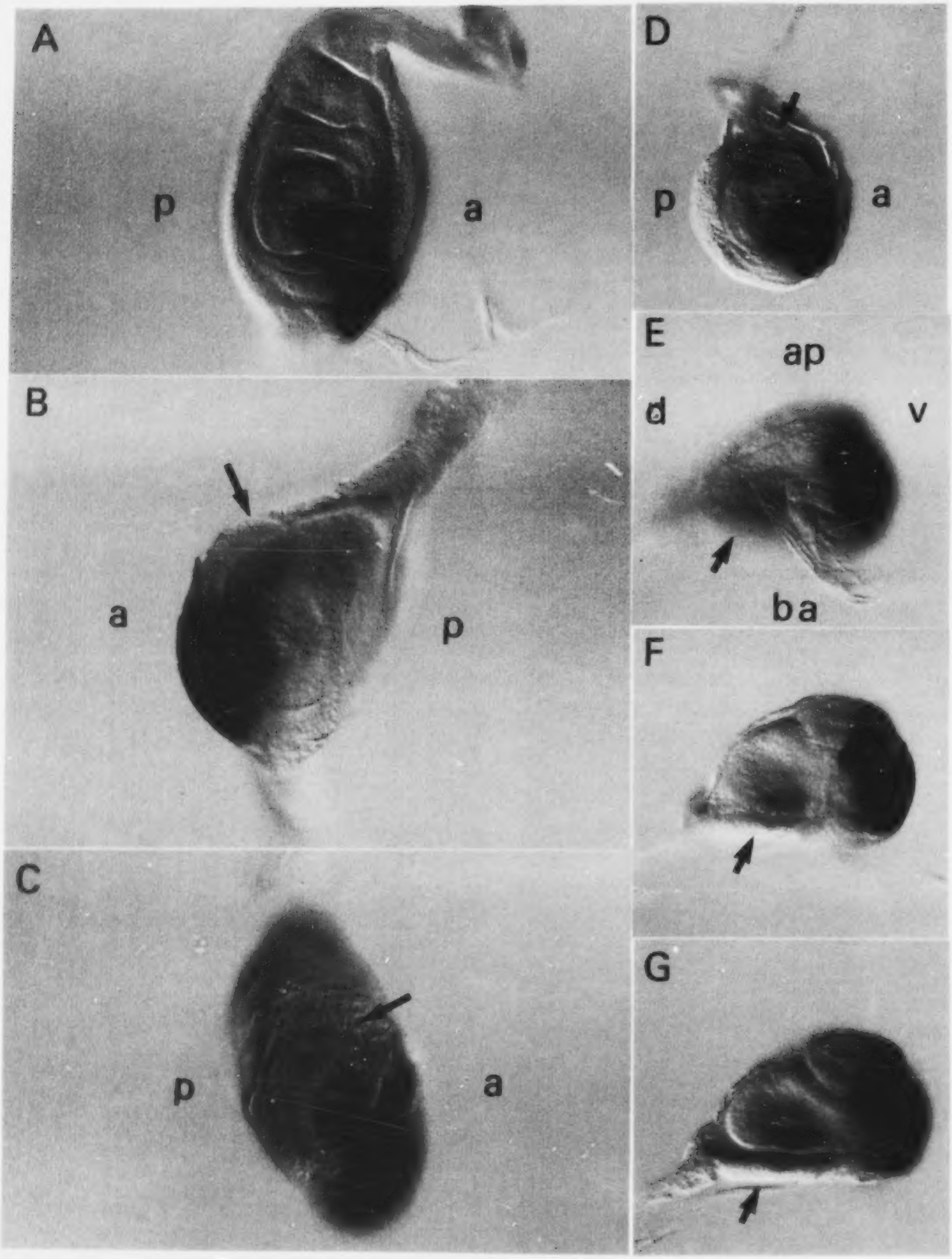
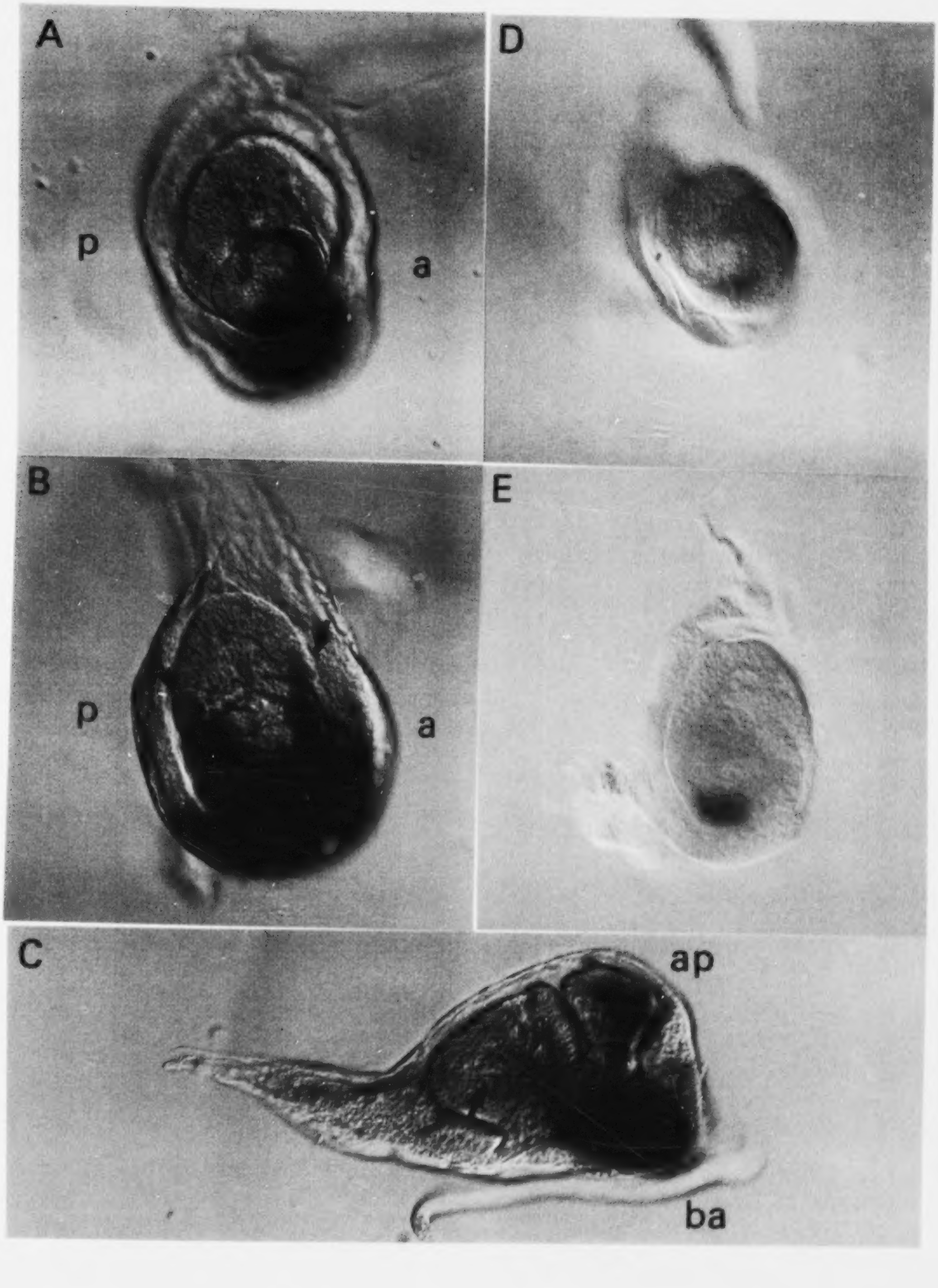

A
p
a
B
a
p
C
p
a
D
p
a
E
ap
d
v
ba
F
G

Table 13: Changes in *wg-lacZ* expression in heat treated *su(f)* leg and wing imaginal discs.

time after heat pulse	larvae scored	leg discs with ectopic *wg-lacZ* patches	wing discs with ectopic *wg-lacZ* patches
0 h	14	0/84	0/28
6 h	19	3/114	0/38
18 h	18	4/108	0/36
24 h	21	9/126	0/42


A
p
a
D
B
p
a
E
C
ap
ba

Harvard Medical School). After treatment in *su(f)*, H-15 spreading was often observed in both the anterior and posterior compartments.

V.2 Time course of enhancer-trap and segment polarity gene expression in cultured leg disc fragments

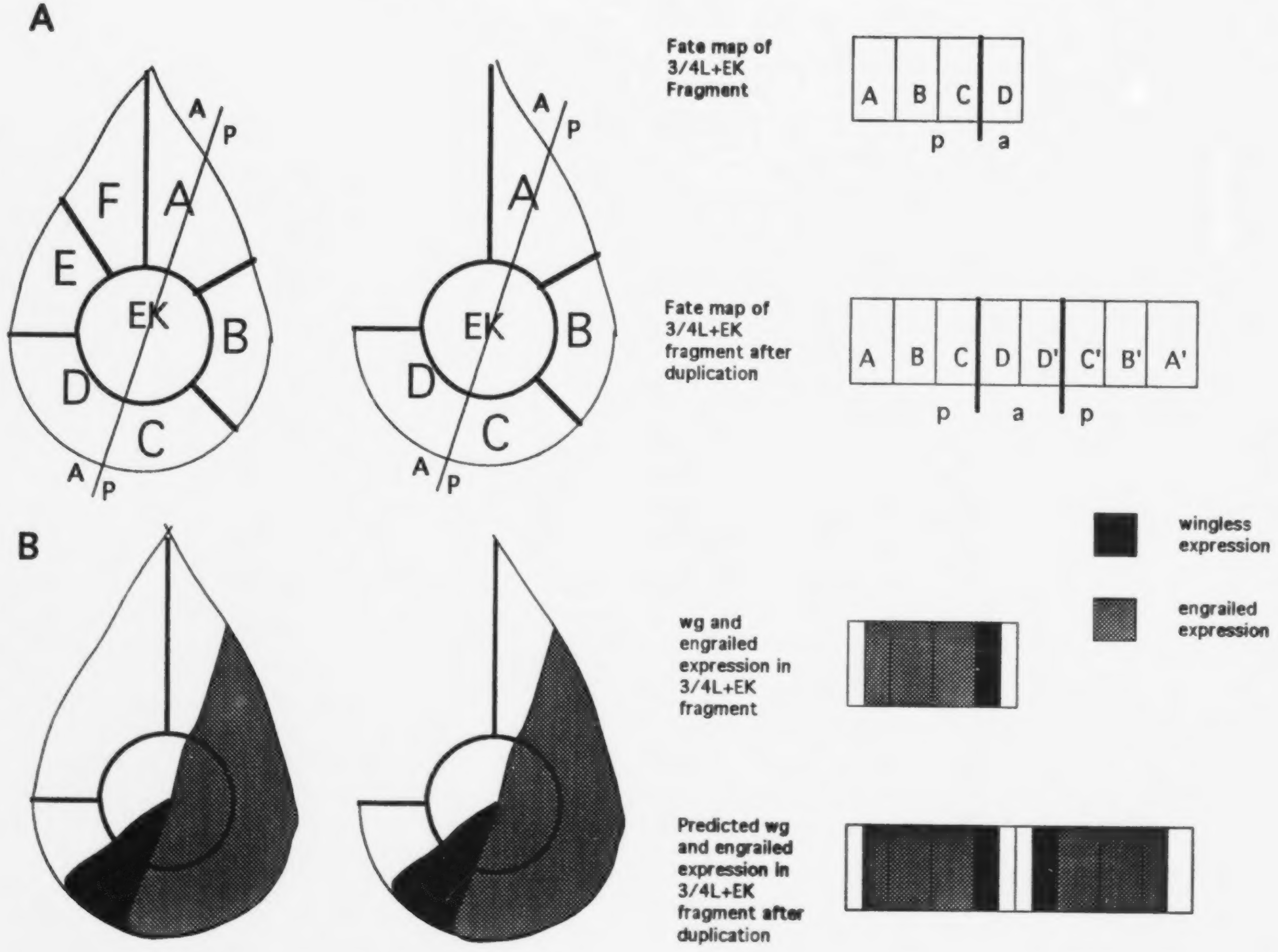
V.2.1 *Wingless* and *engrailed* expression in cultured leg disc fragments

To assist interpretation of expression in the *su(f)* cell-lethal mutant, the expression of *wg-lacZ* and *engrailed* was also examined in cultured leg imaginal disc fragments. The "three-quarter-lateral-plus-endknob" fragment (3/4L+EK) (Abbott et al 1981; Karpen and Schubiger 1983; Kiehle and Schubiger 1985) was chosen for this experiment (see Figure 32). This fragment is useful for several reasons: 1) It yields duplications very similar to those caused by the *su(f)* cell lethal mutation, thus providing a potentially informative comparison; 2) The process of duplication is very well characterized and it has been determined by clonal analysis and labelling of S-phase nuclei, that the blastema is derived mainly from cells from the horizontal edge of the fragment (Abbott et al., 1981; Kiehle and Schubiger, 1985). Clonal analysis shows that these cells, which are from the anterior compartment at a lateral level in the dorsal/ventral axis, can give rise to dorsal, ventral anterior or posterior cells in the duplicate (Abbott et al., 1981). Thus the blastema, comprised of cells which do not normally express *wingless* or *engrailed*, should give rise to the *wingless* and *engrailed* expressing domains of the duplicate. Figure 32 shows the expression of *wg* and *en* superimposed on a wild type fate map and the fate map of a duplicate showing the predicted pattern of *wg* and *en* expression in an idealized duplicated leg disc.

Prothoracic leg discs were dissected from Canton-S or *wg-lacZ*/CyO wandering third instar larvae and fragmented (as shown in Figure 32) to produce the 3/4L+EK fragment. These were injected into adult females hosts and cultured *in vivo* for up to seven days. At intervals, host abdomens were dissected, stained with anti-β-galactosidase and /or the *engrailed* antibody and the cultured discs recovered for examination. Fragments were often quite irregular in morphology and also tend to become elongated in the apical/basal axis, making it very difficult to mount them successfully on microscope slides. The fragments were, therefore, first scored with a dissecting stereomicroscope (magnification 200x) while floating in petri dishes. This allowed the fragments to be viewed from various angles. This greatly aided in the interpretation of both the extent of healing and the staining. The fragments were then mounted under cover slips and photographed with higher resolution using a compound photomicroscope.

The fragments were scored for the extent of healing and for the expression of the *wg-lacZ* and/or the *engrailed* protein. Several patterns of wound healing and staining were seen and these could be correlated with a time course. The fragments were scored as **not healed** if there was no apparent curling over of the horizontal and vertical edges of the wound; they were scored as **partially healed** if the edges had begun to curl over but did

Figure 32: 3/4L+EK Fragment. A: Diagram of first leg disc showing location of cuts generating the 3/4L+EK fragment. Fate map (structures A-F) and pattern transformation after complete duplication are indicated. A/P boundary is also indicated. (after Abbott et al. 1981). B: Domains of expression of *wingless* and *engrailed* in 3/4L+EK fragment and predicted pattern of expression following complete duplication.


A
F
A
E
EK
B
D
C
A
P
A
P
A
EK
B
D
C
A
P
A
P
Fate map of 3/4L+EK Fragment
A B C D
p a
Fate map of 3/4L+EK fragment after duplication
A B C D D' C' B' A'
p a p
B
wingless expression
engrailed expression
wg and engrailed expression in 3/4L+EK fragment
Predicted wg and engrailed expression in 3/4L+EK fragment after duplication

not seem to be in contact with one another; and **healed** if the the two edges were in direct contact with one another. None of the fragments cultured for 6 hours showed any evidence of healing (0/7) and virtually all of the fragments cultured for 1 day had at least partially healed (8/9). The majority of fragments cultured for three days or longer had healed completely (19/21).

Concomitantly, several changes were observed in the expression of *wg-lacZ*. These were found to correlate well with the extent of wound healing. Fragments with no change in *wg-lacZ* expression generally also had not initiated wound healing. These correspond to the **Type I** fragments in Table 14. In fragments recovered later, the *wg-lacZ* expression extended part way towards the horizontal cut edge of the fragment. These fragments (**Type II**) were almost all partially healed (9/10). In fragments classified as **Type III**, the *wg-lacZ* expression extended all the way to the horizontal edge of the wound. Most of these fragments (12/15) were completely healed. The remaining fragments appeared partially healed. **Type IV** fragments had all completely healed and were characterized by a discrete ectopic patch of *wg-lacZ* expression near the site of the healed wound. Examples of types I, II, and III fragments are shown in Figure 33 and a type IV fragment shown in Figure 35.

The distribution of these four types among fragments recovered after different culture periods indicates a progression of stages in pattern duplication (Table 14). In addition to the correlation of *wg-lacZ* expression with the extent of wound healing, fragments recovered after longer periods of *in vivo* culture tend to be composed mainly of types III and IV. Furthermore, in some of the type III fragments, the *wg-lacZ* expression between the original expression and the expression at the site of the healed wound was reduced in intensity. This probably represents a transition from the continuous Type III to the Type IV ectopic patch of *wg* expression which I interpret as a completed pattern duplication.

The expression of engrailed was also examined in 3/4L+EK fragments, either as fragments stained only with anti-*en / inv* or in double labelled fragments stained for anti-β-galactosidase as well. In general, the double stained fragments were more difficult to interpret than single labelled fragments. Since the staining reaction was carried out while the fragments were still implanted in the host abdomens, it was difficult to judge when adequate staining had occurred. It was generally more difficult to discern limits of the *engrailed* staining than that for *wingless* staining. A smaller number of 3/4L+EK fragments were stained only with anti-*en/inv*. No single *engrailed* labelled fragments were cultured for less than two days. None of the fragments cultured for two days (n=6) had any evidence of ectopic *engrailed* expression, except in the peripodial membrane in these fragments seemed stained more intensely than in the discs from the control larvae included in the same staining reaction. In two of five fragments cultured for three days ectopic *engrailed* expression was seen near the site of wound healing. Similarily two of six fragments cultured for four days had apparent ectopic patches of *engrailed* expression.

Table 14.: Type of *wg-lacZ* expression in 3/4 lateral prothoracic leg discs cultured for varying lengths of time.

Days in culture Fragment Class	1/4	1	2	3	4	5	6	7
1	7	1	-	-	-	-	-	-
2	-	8	2	-	-	-	-	-
3	-	-	3	1	7	2	2	-
4	-	-	3	1	3	2	2	1
poor morphology	3	2	5	3	-	1	-	-
Σ	10	11	13	5	10	5	4	1

Figure 33: Patterns *wg-lacZ* expression in 3/4L+EK first leg disc fragments cultured *in vivo*. A: Apical aspect of a control *wg-lacZ* first leg disc immunostained with anti-β-galactosidase to detect *wg-lacZ* (DAB-Ni, dark brown signal) and anti-engrailed/invected (DAB, red brown signal). Fragments in panels B-D are stained only with anti-β-galactosidase B: Apical aspect of a **Type I** *wg-lacZ* 3/4+EK fragment stained 6 hours after injection. No healing or change in expression is apparent C: Apical aspect of a **Type II** *wg-lacZ* 3/4L+EK fragment stained one day after injection. Staining in this fragment is extending towards the site of the partially healed wound. D: Anterior aspect of a **Type III** *wg-lacZ* 3/4L+EK fragment stained four days after injection. The staining in this fragment is extended all the way to the healed wound, and can be seen on both sides of the "scar" of the wound (arrow). The endknob (EK) is indicated in all fragments as is the site of the wound (solid arrow) and the direction of wound healing of the horizontal edge (open arrow). Anterior (a), posterior (p), dorsal (d), ventral (v), apical (ap), and basal (ba) are also indicated.

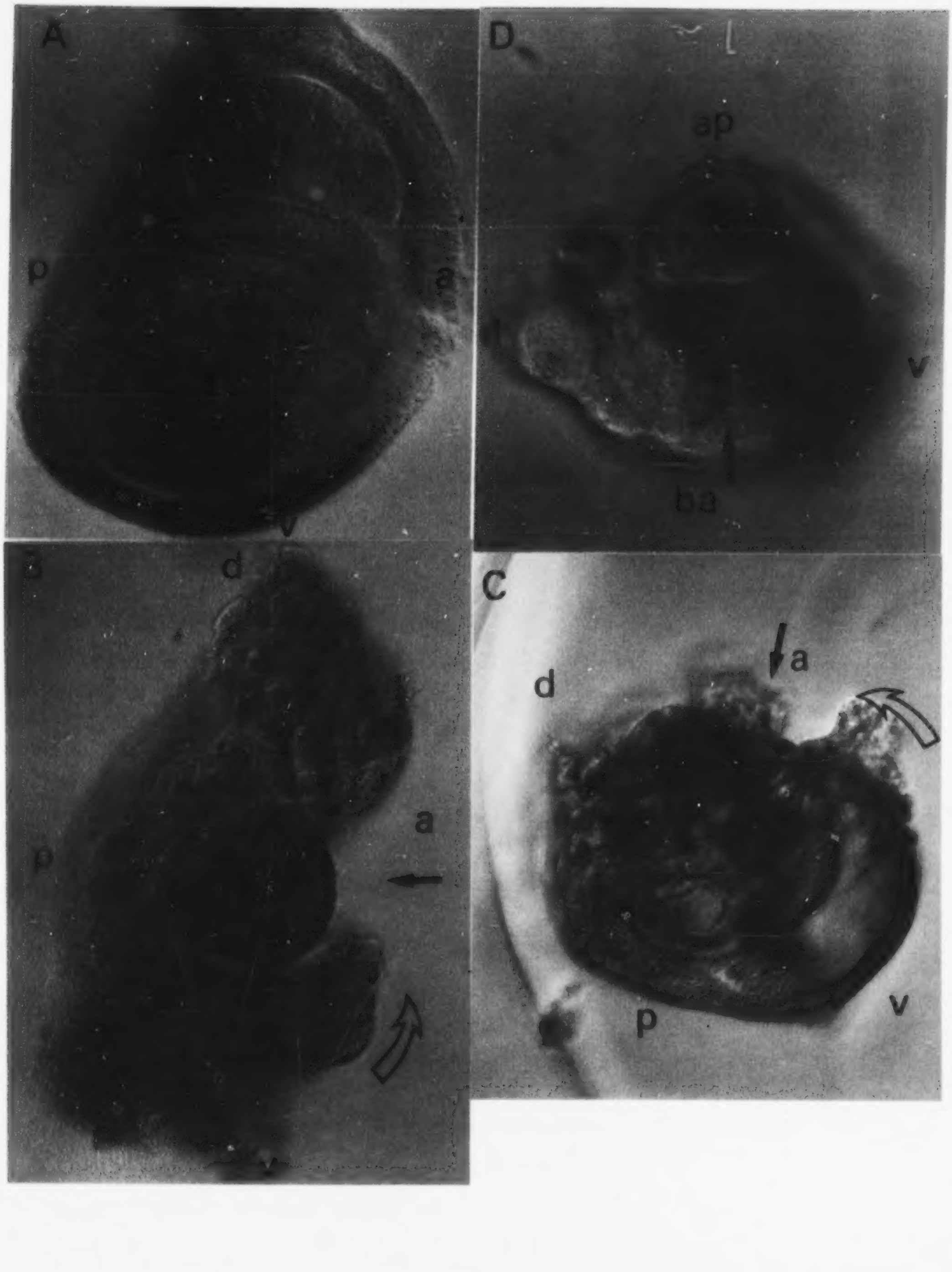

A
p
a
v
D
ap
v
ba
B
d
p
a
v
C
d
a
p
v

Figure 34 shows several planes of focus of a 3/4L+EK fragment cultured for 4 days. Panel C is a basal plane of focus clearly showing *engrailed* expression in the disc epithelium near the site of the wound. At higher planes of focus (Panels A and B) the *engrailed* expression associated with the wound site is continuous with the *engrailed* staining in the peripodial membrane. The fragment is clearly healed and this morphology is either Type III or Type IV where *wg-lacZ* expression is generally found at the wound site. The expression of *wingless* in the wound occurs as early as after 2 days of culture and *engrailed* expression is initiated after 3 days. Thus both *wingless* and *engrailed* are likely to be expressed at the site of wound healing after three days in culture.

This was demonstrated directly in some type III and type IV *wg-lacZ* 3/4L+EK fragments labelled with both anti-β-galactosidase and anti-*engrailed*. No ectopic *engrailed* staining was seen in double labelled fragments cultured for 2 days (0/6). Ectopic *engrailed* staining was seen in four of sixteen double labelled fragments cultured for 3 to 7 days. All four fragments exhibiting the extra patch of *engrailed* expression were Type IV fragments (4/8). Figure 34 shows one such fragment. This type IV fragment has a clear ectopic patch of staining near the site of the healed wound. Higher magnification of the fragment shows clearly that the expression is composed of two groups of cells which meet at a sharp border. One group is stained red-brown with DAB for *engrailed*. The other group of cells is stained dark brown with DAB-Co for the *wg-lacZ* expression. The circumferential sequence of expression is precisely that predicted for a mirror image duplication of the original fragment as shown in Figure 32.

The similarities between the expression of *wg-lacZ* following heat treatment in the *su(f)* mutant and in the duplicating 3/4L+EK fragment are quite striking (compare Figures 30 with 33 and 35). This provides further support for the idea that there is a shared early mechanism underlying the similar outcomes of pattern regulation in the two experimental systems. The failure so far to find ectopic *engrailed* expression in the *su(f)* mutant is probably due to the limited time course examined in my experiments, as discs from larvae allowed to progress as long as 48 h after a heat pulse do show an ectopic patch of *engrailed* expression at the expected site (S. Scanga, pers. comm.).

V.2.2 Expression of enhancer-traps in cultured leg disc fragments

The expression of G-45 was also assessed in 3/4L+EK fragments. Fragments were cultured for one to five days, stained in the host abdomen and recovered. Heads from *wg-lacZ*/CyO larvae were included in the antibody reactions in order to monitor the extent of the staining reaction. The reaction was stopped when the imaginal discs became highly overstained as the *wg-lacZ* line stains much more strongly than G-45. Staining was only observed in G-45 fragments cultured for one day (3/12). Figure 36 shows a G-45 fragment stained after one day in culture. The staining is restricted to the horizontal edge of the wound which is partially healed. This region of the 3/4L+EK fragment has been demonstrated to give rise to the cells of the blastema (Abbott et al 1981;

Figure 34: Expression of *engrailed / invected* in a 3/4L+EK leg disc fragment. Panels A-C are different focal planes of a 3/4L+EK Canton-S leg disc fragment cultured in vivo for 4 days and immunostained with anti-engrailed/invected. The approximate location of the ventral end of the a/p compartment boundary is indicated (p/a) and the fragment is oriented with its apical surface uppermost. A: Top focal plane. In this plane of focus the peripodial membrane of the disc is in focus. *engrailed* staining in the peripodial membrane extends from the posterior compartment towards the region of wound healing (between the open triangles). The limits of staining in focus is indicated by the arrows. B: Middle focal plane. The extent of staining overlaps that shown in panel A showing that the expression is continuous with the expression in the peripodial membrane. C: Basal plane of focus. Staining is in a group of cells in the healed wound. Again the extent of staining at this level (arrows) overlaps that of the level above. This shows that the ectopic staining in the healed wound is continuous with the staining in the peripodial membrane.

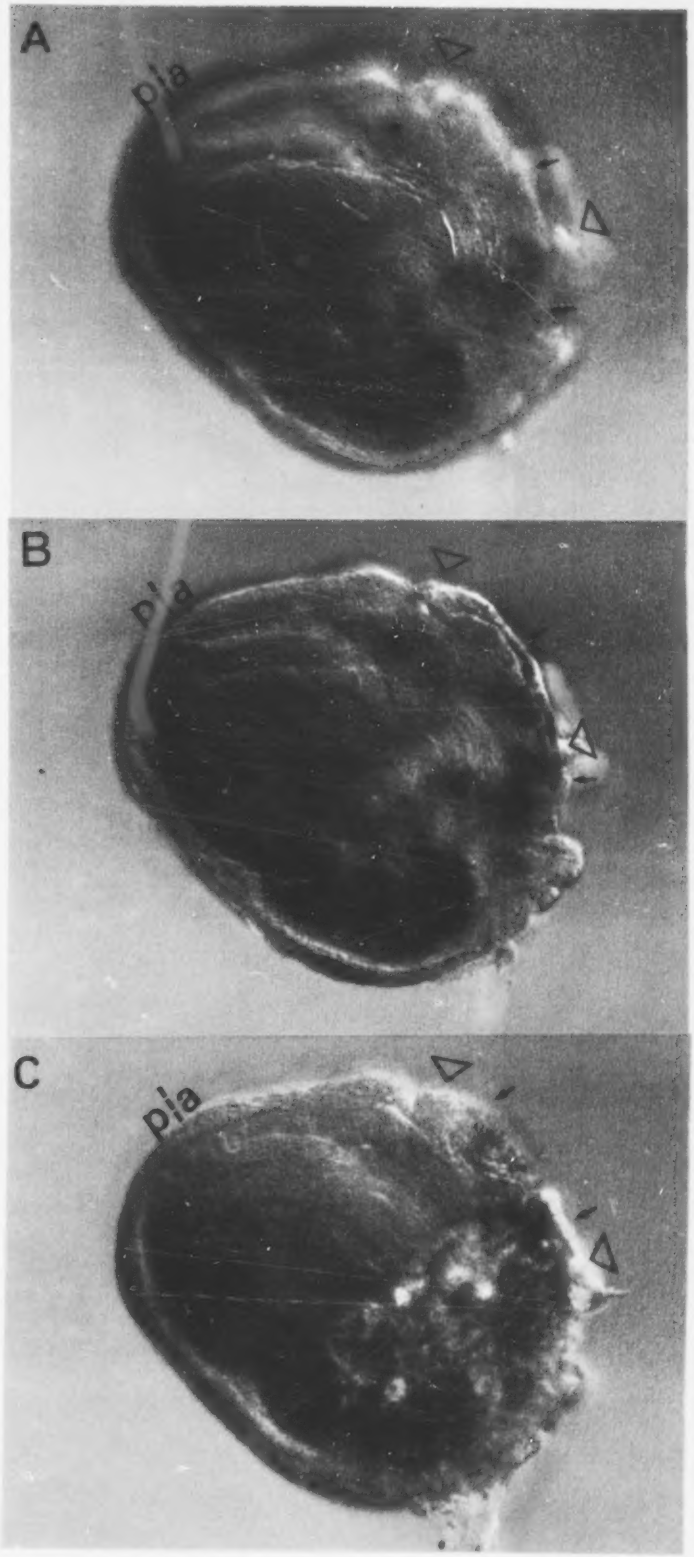

A
pia
B
pia
C
pia

Figure 35: A: Expression of *wingless* and *engrailed/invected* in a type IV *wg-lacZ* 3/4L+EK fragment cultured in vivo for 5 days. Arrows indicate novel patches of *wingless* (dark brown) and *engrailed* (red-brown) expression. B: Higher magnification of fragment in A. Contiguous expression patches of wg and en/inv expression apparently meet at a sharp boundary(......).

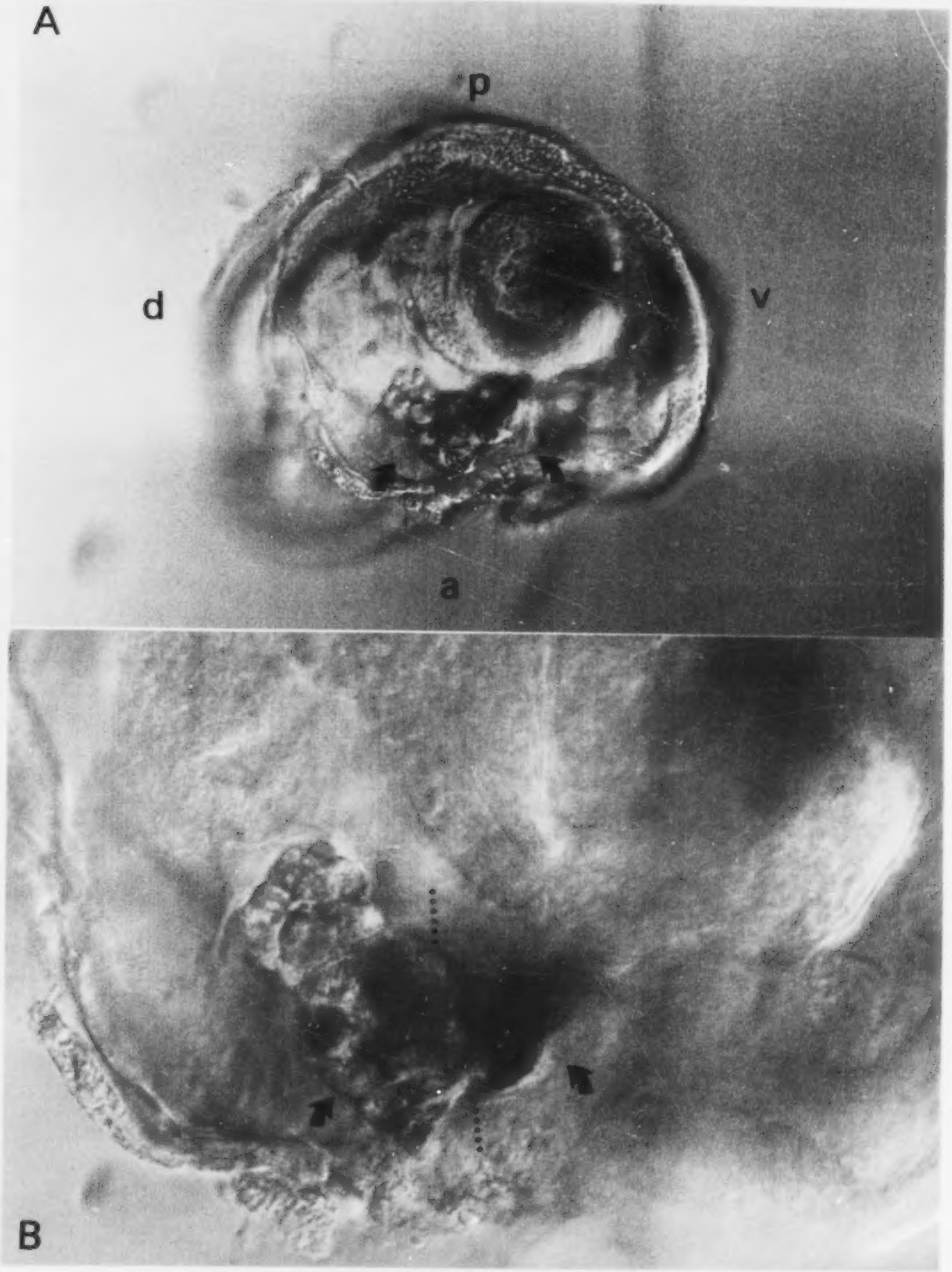
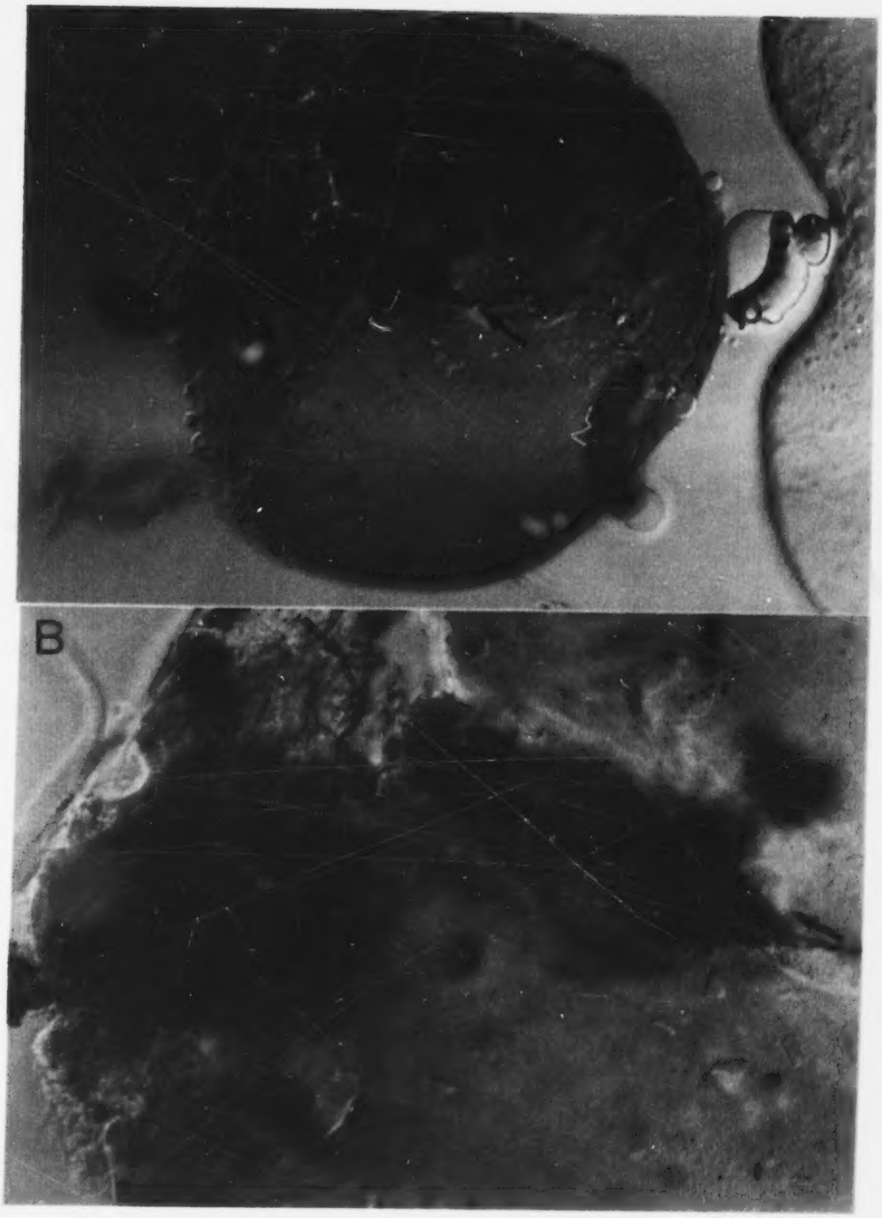

A
p
d
v
a
B

Figure 36: Expression of G-45 in a 3/4L+EK fragment cultured in vivo for one day. A: Immunostaining for anti-β-galactosidase is restricted to the ventral side of the wound (arrows). B: Higher magnification of fragment in A.The fragment is mounted with the apical surface up. Anterior (a), posterior (p), and ventral (v) are indicated.


B

Kiehle and Schubiger 1985). Thus, the expression of the enhancer-traps precedes the expression of *wingless* and *engrailed* in both su(f) and surgically produced disc fragments.

V.4 Expression of genes adjacent to regeneration-induced enhancer-traps following cell death in the *su(f)* background

In order to determine if the enhancer-traps were detecting the expression of adjacent genes, molecular probes for several adjacent transcription units were examined for altered expression in the treated *su(f)* discs. The results are summarized at the end of the chapter in Table 15.

V.4.1 Expression of the *dpp* and *tnd* cDNAs in *su(f)* regenerating discs

The expression of transcripts homologous to cDNAs from the region of the E-32 insertion were examined in *su(f)* imaginal discs which had been subjected to a 48 h heat pulse. Digoxigenin (DIG) labelled RNA complementary to the *dpp* message was synthesized with T7 RNA polymerase from the plasmid H1 5'->3' which is the KP1 cDNA described by St. Johnston and colleagues (1990) subcloned into Bluescript SK+(Stratagene). H1 5'->3' was linearized using the resriction enzyme *Hin*d III. The probe was size reduced as described in the Methods. The hybridization to both control and treated *su(f)* imaginal discs was found to be quite variable with quite high background. Most of the background staining seemed to be in the peripodial membrane. In approximately 10 % of the imaginal leg discs assayed 3, 6 or 12 hours following a heat treatment, there was ectopic expression in the anterior ventral disc epithelium (see Figure 37 B, C for examples). This was different than in control leg discs which had strong staining along the dorsal a/p boundary and weaker staining along the ventral a/p boundary (see Figure 37 A) as previously described (Masucci et al., 1990). Thus, *dpp* expression does appear to be altered at least in a subset of treated discs.

A probe complementary to the *tnd* transcript was synthesized from plasmid NN9SK+, which contains a 3.2 kB *tnd* cDNA inserted in the Bluescript SK+ vector (Stratagene) (R. Blackman pers. comm.). NN9SK+ was linearized with restriction enzyme *Sal* I and digoxygenin-labelled RNA was synthesized using T3 RNA polymerase, and size reduced as described in the Methods. No hybridization was detected in control imaginal discs. All imaginal discs assayed 4 h after 48 h heat pulse displayed *tnd* hybridization. In several treated imaginal wing discs, a faint signal was observed apparently at the a/p boundary as well as in other parts of the disc (see Figure 38). This pattern is very similar to that seen in E-32 discs treated in the *su(f)* cell-lethal background (not shown). *tnd* expression was not seen in discs 24 h after a 48 h heat pulse, implying that *tnd* is transiently expressed following the heat treatment.

BS 3.0 is a P-element construct in which a portion of the *dpp* disk enhancer region drives the expression of of the *lacZ* construct in a pattern similar to *dpp* in imaginal discs (Blackman et al., 1991; Raftery et al., 1991). BS 3.0 was crossed into the the *su(f)* background. It was found that the normal control expression was not detectably altered in any of the discs from a sample

Figure 37: Expression of *dpp* RNA in control and treated imaginal discs. All disc were probed with H1 5'->3' probe A: Canton-S leg imaginal disc. B: *su(f)* leg disc 3 h following a 48 h heat treatment. arrow indicates ectopic expression in the anterior lateral portion of the disc. C: *su(f)* leg disc 8 h following a 48 h heat treatment. Arrow indicates ectopic expression in the ventral portion of the disc.

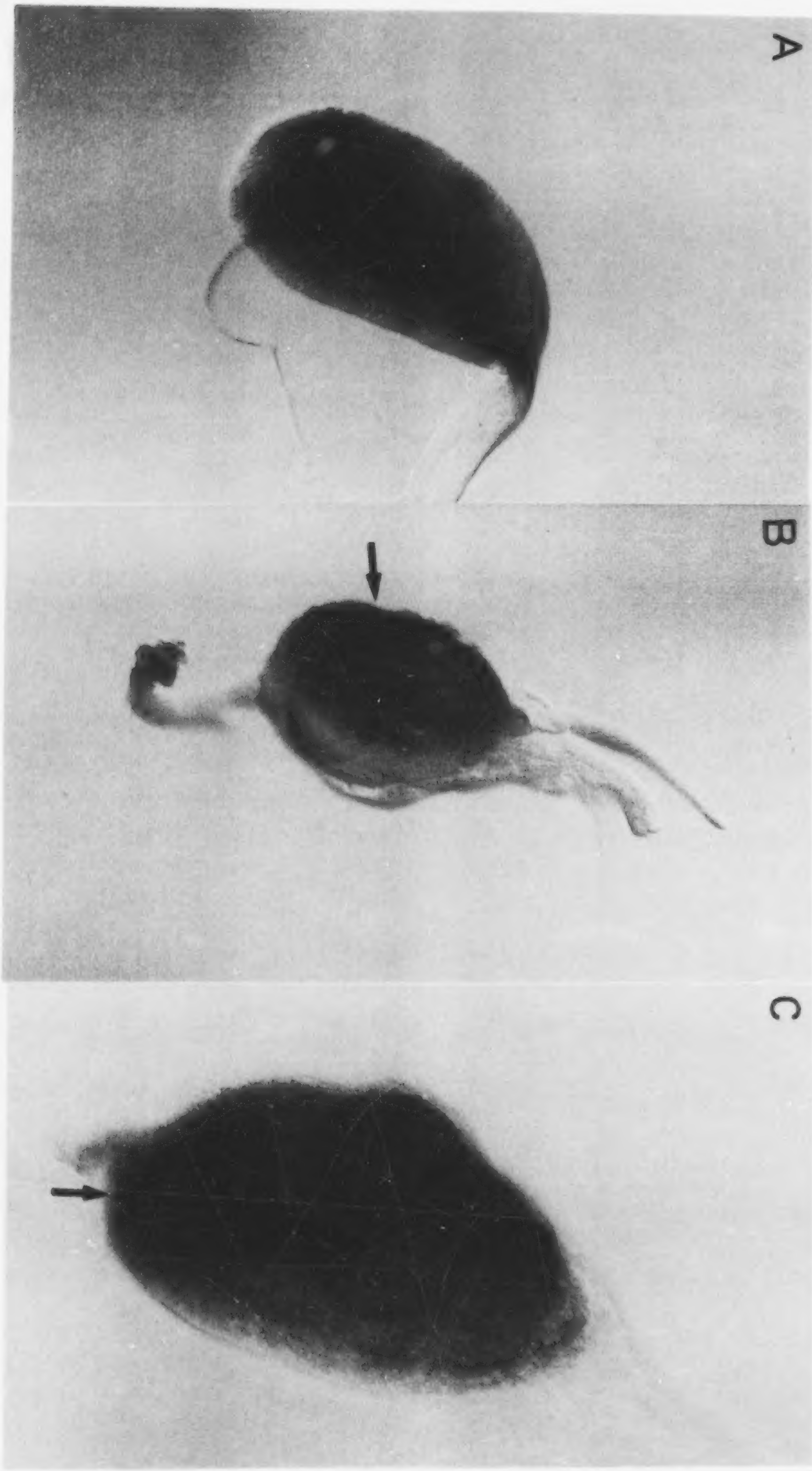
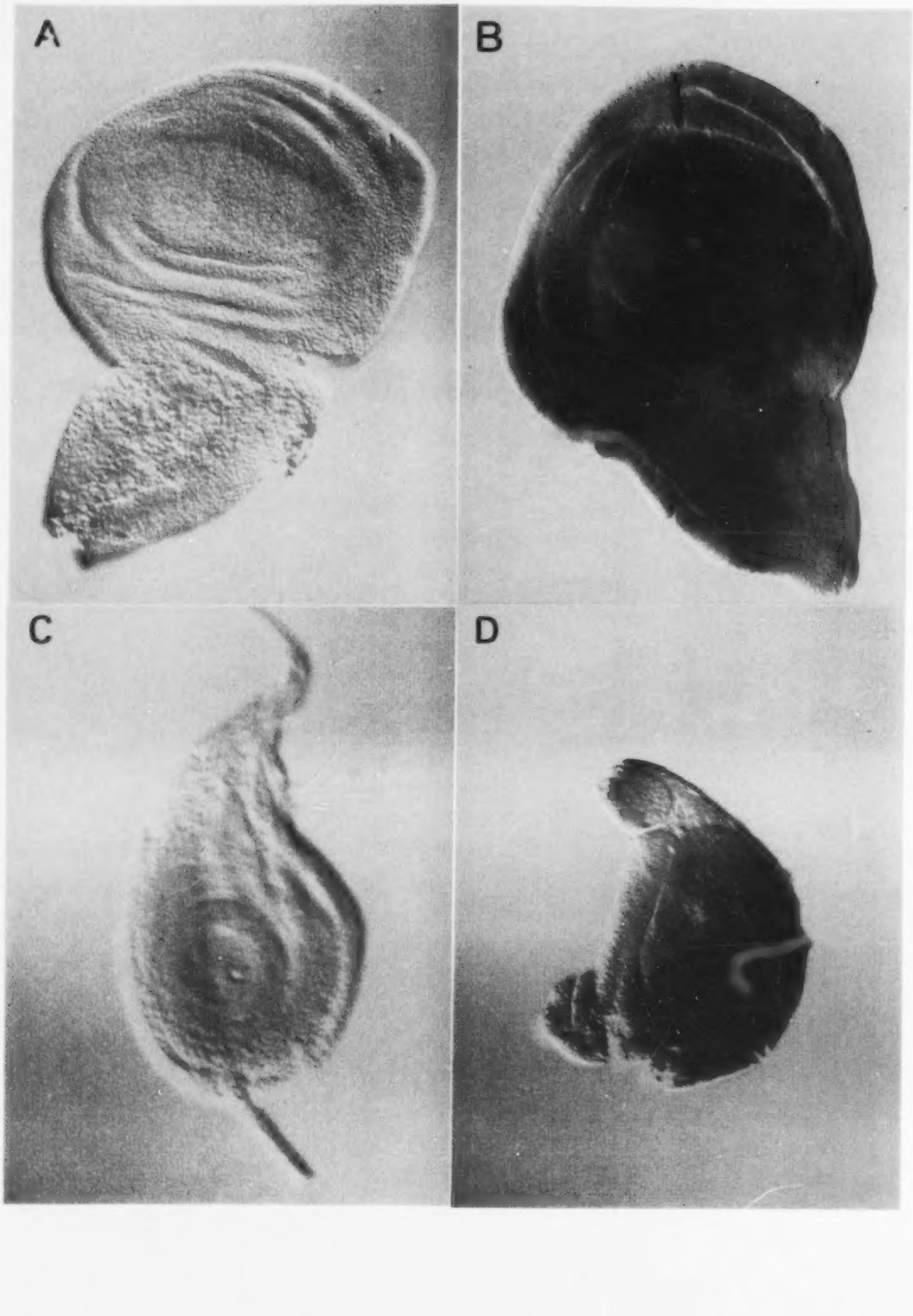

A
B
C

Figure 38: Expression of *transcript-near-decapentaplegic* in control and *su(f)* imaginal discs. All discs hybridized to the *tnd* probe NN9 A: Canton-S control wing disc. B: *su(f)* wing disc 3 h following a 48 h heat treatment. Expression near the location of the a/p boundary is indicated with an arrow. C: Canton-S control leg disc. D: *su(f)* leg disc 3 h following a 48 h heat treatment.


A
B
C
D

of approximatetly 10 larvae, 6 h following a 48 h heat pulse in the *su(f)* background. All E-32 discs in larvae treated in a similar manner have ectopic β-galactosidase staining.

Thus it seems that while the expression of *dpp* may be altered in a subset of treated *su(f)* imaginal discs, the expression of E-32 more closely resembles the expression of *tnd*. The expression of E-32 in cultured wing disc fragments occurs only when cells from the anterior and posterior compartment boundaries come into contact. This strongly suggests that the expression of the enhancer-trap in cultured discs may be due to *dpp*. Cultured wing disc fragments were therefore hybridized to *dpp* and *tnd* probes but no fragments have so far been recovered in several attempts. This may have been due to the more extensive washing during *in situ* hybridization protocol as compared to antibody staining , or perhaps due to other differences in the treatment procedures, for example, the proteinase K digestion.

V.4.2 Expression of the LF-O6 transcript in *su(f)* treated imaginal discs

The cDNA LF-06 is a 3kb clone representing the gene reported by the AD-55 insertion element. Digoxigenin-labelled RNA complementary to the LF-06 message was synthesized with T7 RNA polymerase from the plasmid pLF-06 (linearized with *Xba* I) which contains the LF-06 cDNA in the bluescript KS+ vector (Stratagene). Treated*su(f)* imaginal discs were found to have altered expression when compared to untreated control imaginal discs but the changes were not as extensive as observed for the AD-55 enhancer-trap insertion (not shown).

V.4.3 Expression of the *crumbs* protein in *su(f)* treated imaginal discs

A monoclonal antibody to the *crumbs* protein was provided by Dr. E. Knust, Koln FRG. Comparisons between control and treated imaginal discs show definite differences (see Figure 39). The staining in all treated discs is more disorganized than in the control and virtually all treated leg discs examined in over 25 larvae had some ectopic expression of the protein. The differences were not as pronounced as those seen for the enhancer-trap B-82 but it has been argued that the *crumbs* protein has a very short half life (Tepass et al., 1990) and so the differences may be due to effects of the perdurance of β-galactosidase relative to the *crumbs* protein.

V.4.4 Expression of transcripts detected by the genomic DNA flanking the G-45 insertion

Digoxigenin-labelled RNAs were transcribed from both strands of the rescued G-45 flanking DNA which had been subcloned into Bluescript SK-. The orientaion of the two probes relative to the rescued G-45 flank are shown in Figure 40. The two probes were hybridized to control larvae and to larvae 0, 4, 8, and 12 hours following a 48 h heat treatment in the *su(f)* background. The T3 transcript was found to detect a message which was expressed in imaginal discs 0 hours following the heat treatment, weakly in discs 4 hours following the

Figure 39: Expression of *crumbs* protein in control and *su(f)* treated discs. A: Canton-S control first leg disc showing uniform *crumbs* expression (except for in the outer fold of the disc, indicated by the dot, where there is no expression). Apparent rings of expression (arrows) are caused by the apical distribution of the crumbs protien (Tepass and Knust, 1990) and the folding pattern of the leg disc. B: *su(f)* first leg disc pair, 12 h following a 48 h heat treatment. Arrows indicate patches of increased *crumbs* staining.

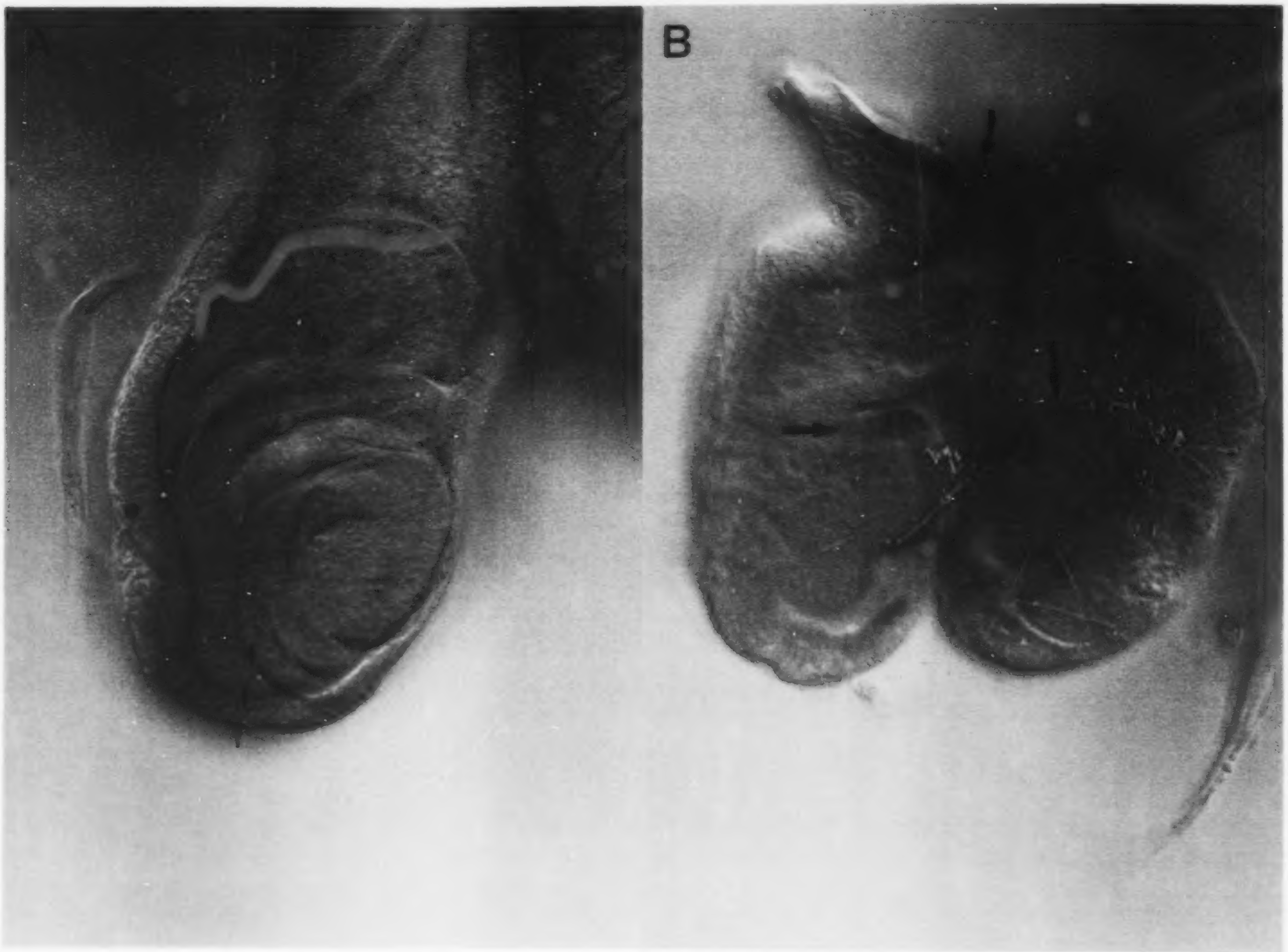
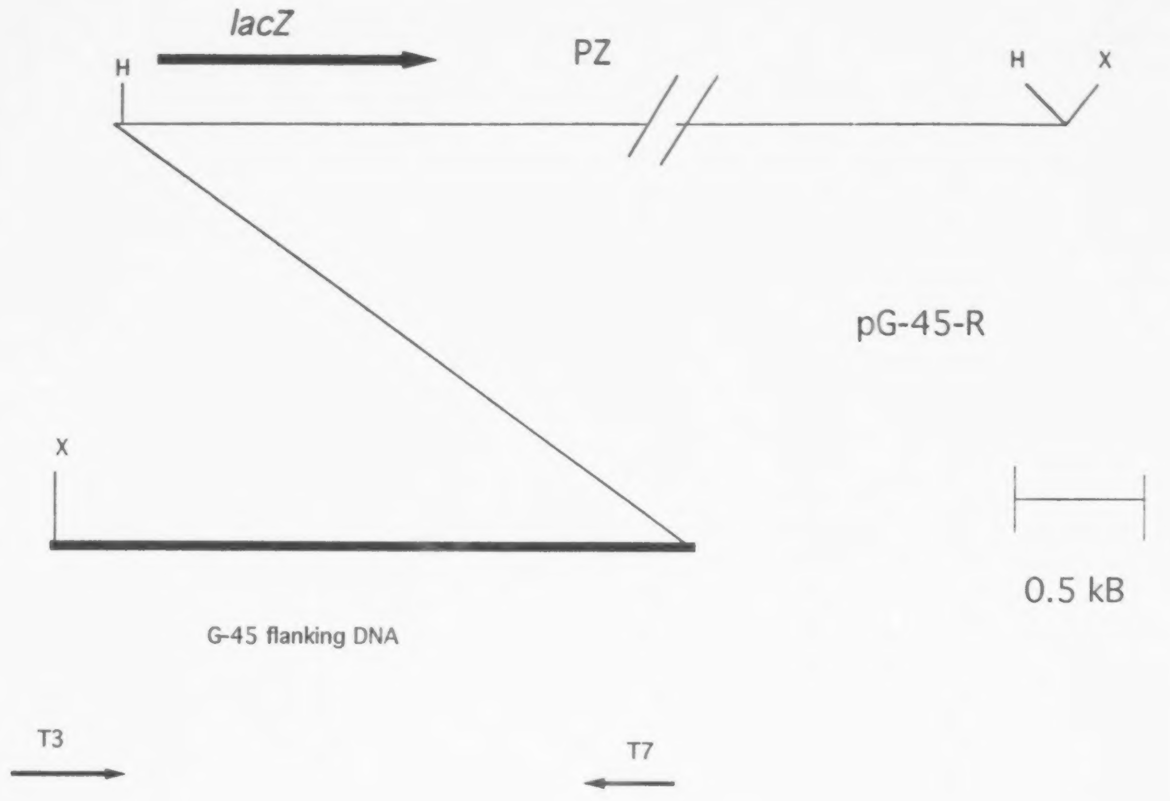

A
B

Figure 40: Restriction map of pG-45-R. The 5'–> 3' orientation of the T3 and T7 RNA probes synthesized from the G-45 flanking DNA are shown in relation to the enhancer-trap.


lacZ
H
PZ
H
X
pG-45-R
X
0.5 kB
G-45 flanking DNA
T3
T7

heat treatment, but not at all in control discs or discs 8 or 12 hours folllowing the heat treatment (see Figure 41). This is very similar to the G-45 enhancer-trap time course described above (see Table 12 and Figure 31 D, E). It also hybridized to larval brains in a pattern similar to that of the G-45 enhancer-trap expression (not shown). The T7 probe, which is in the same transcriptional orientation as the *lacZ* message in the enhancer trap, detected a transcript restricted to the developing ommatidia in both control and treated imaginal discs. Some weak staining was observed in the antennal segments of some discs from both control and treated discs, but no other disc staining was observed. The pattern of hybridization in the larval brain was found to be distinct from that for the T3 probe and the G-45 transcript.

V.5 Chapter Summary

In this final chapter of results, I have shown that the majority of the selected enhancer-traps are expressed in duplicating discs prior to the ectopic expression of *wingless,* as detected by an enhancer-trap in the *wingless* locus. Both *wg-lacZ* and *engrailed / invected,* were found to be expressed ectopically in cultured leg discs, and the insertion G-45 was found to be expressed prior to both genes in this system. Also, four insertions which are expressed in regenerating discs (B-82, E-32, G-45, and AD-55) were found to have corresponding adjacent wild-type genes which had altered expression in heat treated *su(f)* discs (see Table 15).

Figure 41: Expression of trancripts homologous to the G-45 flanking DNA in control and *su(f)* treated discs. A: Canton-S control discs and brain hybridized with the G-45 T3 RNA probe. Eye antenna disc (e), a leg disc (L) and brain (b) are indicated. B: Heat-treated *su(f)* brain and discs hybridized with the G-45 T3 RNA probe immediately following a 48 h heat pulse. C: Canton-S control eye-imaginal disc hybridized with the G-45 T7 RNA probe. The approximate position of the morphogenetic furrow is indicated (arrow). D: Heat-treated *su(f)* eye-imaginal disc hybridized with the G-45 T3 RNA probe immediately following a 48 h heat pulse. The approximate position of the morphogenetic furrow is indicated (arrow).

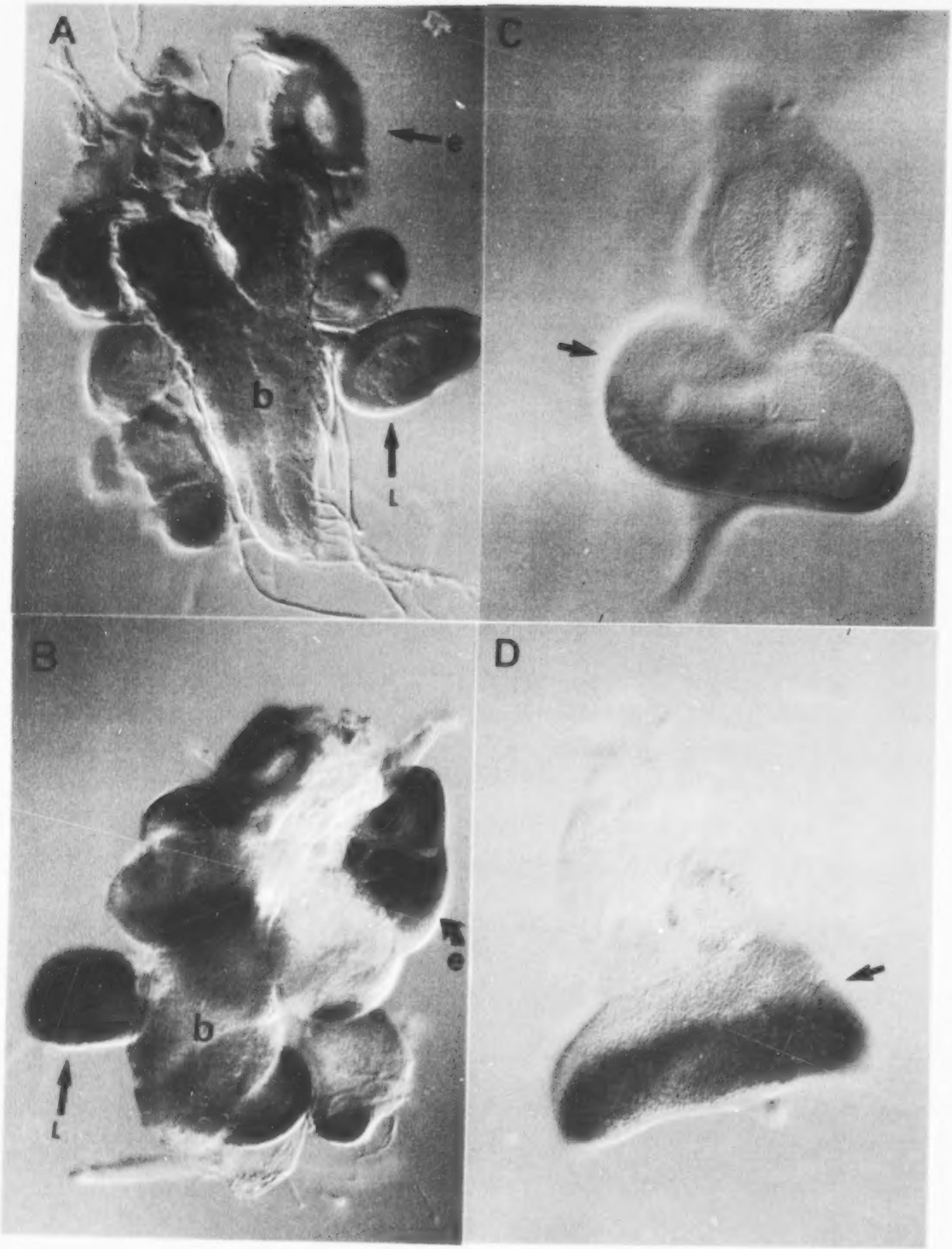

A
e
b
L
C
B
e
b
L
D

Table 15: Expression of genes adjacent to regeneration-induced-insertions in treated su(f) imaginal discs.

Insertion	Probe	Imaginal disc control expression	Heat treated *su(f)* imaginal disc expression
B-82	crumbs antibody	uniform	ectopic patches in all leg discs
E-32	dpp RNA probe	a/p boundary in all discs	ectopic expression in ~10% of leg discs
in	tnd RNA probe	no expression	expression induced all discs
G-45	T7 RNA probe from flanking genomic DNA	behind morphogenetic furrow in eye discs	no change
	T3 RNA probe from flanking genomic DNA	no expression	expressed in all discs
AD-55	LF-06 cDNA	a/p boundary in all discs	broader and irregular a/p boundary expression.

VI. DISCUSSION

Using an enhancer-sensitive P-element construct, I have conducted a systematic screen for genes expressed during imaginal disc regeneration. This is a well studied system, potentially important for elucidating the nature of positional information and its role in pattern formation (Wolpert, 1969). A genetic approach has not been systematically applied to the study of regeneration, due to the difficulty of screening by standard methods for mutations defective in the process. Saturation genetic screens have been used to tremendous advantage in the analysis of several aspects of *Drosophila* development notably oogenesis and segmentation (Nüsslein-Volhard and Wieschaus, 1980; Schupbach and Wieschaus, 1989; Nüsslein-Volhard et al., 1987). Genes critical to a particular stage of development can be identified by screening for mutants disrupted for the process. Such a screen for regeneration mutants presents several difficulties. Regeneration must be induced in some manner so that the effect of mutations on the process can be assessed. If earlier developmental mechanisms are re-utilized during regeneration, regeneration mutants would die early in development. Therefore, to ensure an unbiased sample of the genes involved it would be necessary to screen for conditional mutations. Furthermore, it would be difficult to distinguish mutations defective specifically for regeneration from mutations in loci required for general functions such as cell proliferation (Shearn et al., 1971; Shearn and Garen 1974; Kiss et al., 1976; Gatti and Baker, 1989). Previous studies have suggested that these may number in the hundreds. Genes which are only required during regeneration could be in principle detected by a standard screen, but it is unknown if any genes with such a restricted function exist.

Because it identifies genes on the basis of their regulation, the new enhancer-trap technology (O'Kane and Gehring 1987) presented an opportunity to search directly for genes with altered expression in regenerating discs. Since expression is detectable as a dominant phenotype, the insertions could be screened as heterozygotes over a wild type chromosome to complement any essential functions which might be disrupted by an insertion. Thus I could identify a sample of loci unbiased with respect to their possible functions at earlier stages of normal development. Enhancer-trap insertions may also identify redundant functions or genes with phenotypes which are difficult to discern. Finally, the design of enhancer-traps facilitates cloning and mutagenesis based on plasmid rescue and marker-loss, respectively (Wilson et al., 1989; Fasano et al., 1991).

VI.1 Design and Validation of Screens

The initial screen made use of a temperature-sensitive cell-lethal mutation to induce disc lesions subsequently repaired by regeneration (Russell, 1974). I screened for altered *lacZ* expression 24 hours after the end of the heat treatment. Earlier studies have shown this time point follows detectable cell death (Clark and Russell, 1977), and precedes the re-initiation of cell division and the restriction to anterior or posterior compartment of marked clones in the regeneration blastema of duplicating leg discs (Girton and Russell 1980, 1981).

Thus this time point may coincide with early steps in regeneration important for specification of the new pattern. To eliminate false positives induced early by the cell lethal mutation before cell death ensues, I re-screened during the heat treatment when the disc cells are just beginning to die, and therefore prior to initiation of regeneration. As a further tactic I reasoned that γ-radiation might induce a different set of cell-death genes (reviewed by Raff, 1992), but that the resulting disc lesions would be repaired by a common regeneration mechanism. Thus real positives would be induced also by γ-ray treatment. Application of these secondary screens yielded the four classes of enhancer-trap insertions shown in Fig. 7.

A true regeneration gene should be expressed ectopically in a regenerating disc fragment early during *in vivo* culture. The effectiveness of the screens was supported by the results of this test with disc fragments from the four classes of enhancer-trap lines. Half of the Class I (late *su(f)* and γ-ray induced) insertions tested were ectopically expressed, and all but one of the remaining positives were from Class II (induced by late su(f), not by γ-rays). The substantial frequency of about one third of Class II insertions scored as positive in cultured disc fragments suggests that insertions in some regeneration genes may fail to respond to radiation-induced cell-death under the conditions of my screen. Both cell-lethal and γ-ray induced lesions are thought to be repaired by intercalary cell-divisions, but the early wound healing processes might be different because of the more uniform spatial distribution of the cell-death in irradiated discs (Spreij, 1971). The cell-lethal mutation causes a strongly clustered pattern of cell-death (Clark and Russell, 1977) (e.g. Fig. 5 B). Also, because the discs were assayed at different times after the cell-lethal and γ-radiation treatments, some transiently expressed functions may have been detected only as Class II insertions.

The most persuasive evidence that the loci detected in my screens may be involved in regeneration comes from the expression patterns observed in disc fragments cultured in adult female hosts, where the location of the regenerating cells can be identified. In most of the positive lines from this test, ectopic expression was consistently localized in small numbers of cells at epithelial discontinuities. That these represent sites of wound healing, where the regenerating cells originate may be deduced from the way the disc epithelium is known to fold resulting in closure of the wound (Reinhardt et al., 1977; Reinhardt and Bryant, 1981; Dale and Bownes, 1985). The patterns and numbers of cells at the healed wound which express *lacZ* were similar in some cases to those reported for S-phase cells detected with labelled nucleotides in wing disc fragments (O'Brochta and Bryant, 1987). Figure 9 shows a G-45 wing disc fragment in which approximately 100 β-galactosidase positive cells are in focus on one side of such a discontinuity. O'Brochta and Bryant (1987) found 200 to 450 S-phase cells on both sides of the wound heal in serial reconstructions of similar fragments. The patterns of β-galactosidase staining observed were very similar to those presented in Figure 7 of Bryant and Fraser

(1988) who used antibodies against BrdU incorporated into S-phase cells in whole mount discs.

Based on regulative properties and high mitotic index, Schubiger and Karpen (1983) define cells in this location as a regeneration blastema analogous to those in vertebrate limb regeneration (Goss 1969) . There were differences among the lines identified in this study in the extent and pattern of expression in the vicinity of the wound heal. Nineteen of the lines expressed β-galactosidase restricted to within approximately 10 cell diameters of the site of wound healing. In G-45 (Figure 9) and E-32 (Figure 12), the expression was always restricted to one side of the healed wound whereas other lines (for examples see Figures 12 and 14) had symmetric expression or both symmetric and asymmetric staining that might indicate stages in the evolution of a pattern (AD-55). The amount of staining in similar fragments could also vary from less than 10 cells staining to at least 100. (compare Figure 9 A, C with Figure 14 B). Four other lines (C-76, E-37, F-36, E-60) often had expressing cells more distant from the site of wound healing as well as in the blastema.These differences may reflect real diversity in the roles of the genes in wound healing or establishment of the blastema, but detailed studies will be required to fully define the temporal and spatial expression patterns characteristic of specific insertions.

VI.2 Genes expressed during imaginal disc regeneration

The proportion of all enhancer-traps in which ectopic expression in regeneration was actually demonstrated was 2.8 % (23/826). This represents a minimum estimate of the real proportion as only a selected sample of the insertions were examined in cultured discs for technical reasons. Another estimate of 9.2 % can be derived by calculating a weighted average of the proportions of ectopically expressed insertions found in each class of lines from the screen[*] . This estimate may be biased because the lines tested were not a random sample. Since the sample of enhancer-traps studied here can only represent a fraction of the total possible genomic sites (see Cooley et al., 1988), either of these figures would imply a genomic response of the order of hundreds of genes activated during regeneration. Thus my results indicate extensive novel gene expression at the expected time and putative site of pattern regulation.

Such a massive transcriptional response prompts the question of the specificity of the screen. One possibility is that the response accurately reflects a massive requirement for new gene expression during regeneration. Studies suggest that perhaps hundreds of genetic functions are required for imaginal disc development (reviewed in Shearn, 1977) and it may be possible that some of our insertions represent the redeployment of these functions during regeneration. However, this cannot explain all of the reponse as the expression of many insertions is recruited during regeneration. In my sample, 6 % (15/227) of insertions which are not normally expressed in imaginal discs are expressed in the regeneration blastema. The majority of these (10/15) are not expressed in the embryonic ectoderm and thus could not represent functions required early in disc development which are re-iterated during regeneration.

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[*] The sum of [(the proportion of positives in a given class)(the proportion of lines in that class)] for classes one to five:
$[(4/8)(44/826)]+[(19/58)(156/826)]+[(1/6)(19/826)]+[(0/14)(93/826)]+[0(514/826)]=0.092$. This calculation is based on data presented in Tables 2 and 4 of chapter III. The proportion of positives in class V was assumed to be 0.

Studies of other regenerating systems such as the hydra (Schummer et al., 1992), amphibian limb (Brown and Brockes, 1991) and rat liver (Mohn et al., 1991), have also indicated that there is extensive novel gene expression during regeneration. Mohn and colleagues identified 67 differentially expressed cDNAs in the immediate early response in regenerating rat liver, 14 of which represented novel transcripts. Thus large responses are not unprecedented. Whether or not the expression of these genes or the genes identified by the enhancer-traps in this study has functional significance remains a question.

The enhancer-traps may report irrelevant gene expression brought on perhaps by the pleiotropic effects of widespread remodelling of chromatin states during regeneration. The normally heritable expression patterns of genes encoding states of determination, such as genes of the *Antennapedia* and *bithorax* complexes and putative selector genes such as *engrailed*, are suggested to be determined by special chromatin states (Peifer et al., 1987; Paro, 1990). In this hypothesis, the *Polycomb* group of genes (Pc-G) are thought to mediate the maintenance of the inactive states of certain genes in heterochromatin like domains (Busturia and Morata, 1988; Tiong and Russell, 1990). Thus if these selector genes were to become reactivated during regeneration, it would have to be accompanied by an opening of the closed chromatin state. There is indirect evidence that this must occur. For example the expression of *engrailed* changes during regeneration as seen in this study and as inferred from changes in compartmental commitments during regeneration and duplication (Szabad et al., 1979; Girton and Russell, 1981; Abbott et al., 1981). There are examples of anterior compartment cells, which do not express *engrailed* giving rise to posterior compartment tissue, which presumably does express *engrailed*. Furthermore, the expression of genes in the *Antennapedia* and *bithorax* complexes is likely to be labile during regeneration as well. This is shown by the tendency for fragments to transdetermine to different segmental states during *in vivo* culture (Hadorn, 1965; Schubiger, 1971) especially after dissociation and mixing of cells (Strub, 1977). It is uncertain whether this is a general enough phenomenon to account for the response seen is this screen, however, the products of the Pc-Group genes are chromatin constituents found immunohistochemically at approximately 60-70 sites on the polytene map (Zink and Paro 1989; DeCamillis et al., 1992). This number of sites would probably not be sufficient to explain the extent of the response estimated in this screen, but, it has been suggested that the Pc group and the modifiers of positional effect variegation are functionally related (H. Brock, unpublished). The large group of PEV-modifiers are also thought to influence the stability of heterochromatin (Locke et al., 1988), so it is possible that a re-ordering of chromatin during regeneration could be more general than just in targets of the Pc-group.

Regulation of chromatin structure during regeneration could explain the expression of transcripts near the *decapentaplegic* locus, adjacent to the insertion E-32. Characterization of flanking genomic DNA obtained by plasmid rescue has indicated that E-32 is inserted immediately adjacent to *dpp*, downstream of the 3' disc enhancer and in the 5' untranslated leader of an adjacent transcription unit, *transcript-near-decapentaplegic* (*tnd*) (R. K. Blackman, personal communication). The developmental expression profiles of the *tnd* and *dpp* transcripts are summarized in Table 16 and indicate that the E-32 insertion reports the expression of both *dpp* and *tnd*. In heat-treated *su(f)* imaginal discs, the *dpp* transcript was ectopically expressed in a localized manner in only a subset of discs. The *tnd* message, normally not expressed in discs, was found to be broadly induced in all treated imaginal discs. In some cases, the *tnd* transcript was induced in a stripe in the middle of the wing disc resembling the standard *dpp* expression as well as weakly throughout the disc (see Figure 38). It is therefore possible that the chromatin in the region is reorganized so as to eliminate a domain boundary that normally restricts the influence of the enhancers of each gene to their cognate basal promoters. Thus the restructuring of chromatin in the *dpp* region could cause the fortuitous expression of *tnd*. A subset of these cells might eventually express the *dpp* message depending on positional signals. In this hypothesis, the enhancer-trap would be sensing not the expression of *dpp* per se, but the potential of a cell to adjust its pattern of expression according to cell contacts at the wound heal. This model seems plausible as *dpp* has been proposed to function as part of an organizer of imaginal disc positional information (Gelbart, 1989; Campbell et al., 1993). It seems unlikely that it would be generally expressed during the early stages of disc regeneration since this would incur drastic consequences to the final phenotype of the appendage.

The two messages near the G-45 insertion tend to refute the idea of a general transcriptional upregulation during regeneration, although the data must be interpreted cautiously as the region is incompletely characterized. One of two messages contained within a 2.3 kB genomic DNA fragment is unaffected by the *su(f)* background. The other message, which is transcribed in the opposite orientation, is strongly induced in a pattern similar to *lacZ* in the G-45 strain.

For E-32 and G-45, I have identifed transcripts expressed in a manner corresponding to the expression of the enhancer-trap in regenerating discs. But in order to distinguish between the two models I have proposed to explain the response in the screen, it will be necessary to test the functional requirements for these genes. It is quite possible that *tnd* is purposefully upregulated in regenerating discs and not just fortuitously, as a by-product of *dpp* de-regulation. It is also possible that *tnd* and *dpp* are coincidentally induced in *su(f)* treated and regenerating discs, respectively. In either case, E-32 seems to accurately reflect the distribution of messages transcribed in the region of the insertion. It will be necessary to examine the expression of *tnd* and *dpp* in cultured disc fragments and to test whether there is any requirement for their expression during regeneration using *dpp* and *tnd* mutants.

Table 16: Developmental profile of the E-32 enhancer-trap and the adjacent transcripts *tnd* and *dpp*.

stage	E-32 enhancer-trap	*dpp* message	*tnd* message
larval gonad	male and female gonad[2]	not expressed[2]	male and feamle gonad[2]
adult gonad	testes[2], ooccyte nurse cells and germarium[1]	not expressed[3] nurse cells and germarium[2]	testes, ooccyte
embryo	lateral stripes, segmental spots[2]	dorsal ectoderm, lateral stripes, segmental spots[3]	weak generalized[2]
imaginal discs	a/p boundaries morphogentic furrow	a/p boundaries morphogentic furrow	not expressed
su(f) treated imaginal discs	ectopic expression in all discs[1]	ectopic expression in some discs[1]	ectopic expression in all discs[1]
cultured wing disc fragments	expressed when A and P compartment cells are brought together by wound healing, probably in blastema[1].	not tested	not tested

1) This study. 2) R.Blackman, personal communication 3) Blackman et al., (1991)

The insertion in B-82, a compartment boundary line ectopically expressed in disc fragments, caused a lethal mutation which was revertable by excision of the P-element and allelic to *crumbs* (see Figure 15). However, the *crb* protein is uniformly expressed in imaginal discs (Tepaß and Knust 1990). The *crb* protein contains EGF repeats (Tepaß et al., 1990) and many EGF repeat proteins help mediate cell-cell interactions, either as ligands, surface receptors, and adhesion molecules (Hortsch and Goodman, 1991). Interestingly, the *crb* mutant is a cell-non-autonomous lethal causing the loss of integrity and eventual cell death in several epithelial cell types. It may be required to maintain epithelial continuity (Tepaß and Knust, 1993). This function would likely be very relevant for regeneration. Using an antibody to *crb* (Tepaß et al., 1990), I have shown that the *crb* protein has altered expression in regenerating *su(f)* imaginal leg and eye-antenna discs. This means once more that an enhancer-trap insertion reports a genuine change in gene expression during regeneration. In leg imaginal discs, intense staining is in clusters of cells as might be expected for involvement in wound healing or in signalling between two groups of normally non-adjacent cells during regeneration. The pattern of ectopic expression in the eye-disc was found to be less pronounced but it was clustered to the region in front of the morphogenetic furrow, where the frequency of pattern defects and cell death are highest in the *su(f)* background (Russell, 1974; Clark and Russell, 1977).

A transcript, LF-06 corresponding to another insertion with expression associated with the a/p boundary, AD-55, was examined in treated *su(f)* discs. The distribution of the LF-06 message was found to be altered in expression in the *su(f)* background. However, the transcription unit adjacent to the H-39 insertion gave equivocal results. In general *in situ* hybridization was found to be unreliable with the H-39 probe used, as it gave high background and weak signal. It was impossible to interpret the result in the *su(f)* background. In general, the expression of genes adjacent to the enhancer-traps selected in this study has corresponded to the expression of the enhancer-traps in the *su(f)* background. This indicates that the expression in regeneration must be due to the activity of genuine enhancers and not to artifactual "orphan" enhancers which drive the expression of the reporter genes but have no regulatory role in the wild-type genome.

It will be important to obtain mutations in the genes detected by these insertions to assess their functions, if any, in regeneration and eventually to ascertain their epistatic relationships to see if they comprise a regulatory hierarchy. Mutagenesis by P-element mobilization has been attempted for the insertions G-45, H-39 and H-15. Substantial strain-specific variability was observed in the rates of loss of the ry^+ marker and in the frequency with which lethals were obtained from excisions in the three lines. Characterization of P-element excisions by others suggests that most of the viable ry^- deriviatives will be internal deletions of the P-element (Engels, 1989). The lethal excision derivatives of H-39 are likely to comprise more than one complementation group. Two of the five excisions derived from H-39 (Δ32 and Δ54) (see Figure 29) are definitely multilocus deficiencies and based on the complementation patterns between Δ26, Δ54, Δ215, and Δ3002 (summarized in Table 10), it seems likely that all five are deficient for at least two functions. At least two of the five lethal excisions from the H-15 locus are cytologically visible deletions (D. Long Pers. comm.). Since the putative excisions must delete at least one vital function the size of the recovered deletions may reflect the possibility that the genes reported by the enhancer-traps cannot mutate to an easily detected phenotype. Other mutational screens will be necessary to confirm this. Estimates based on the frequency of reversion of dominant mutations to aphenotypic nulls suggests that as many as 40% of all such genes in *C. elegans* have a wild-type null phenotype (Park and Horvitz, 1986). A possible explanation would be the prevalence of redundant multigene families. All members of such families would be potential targets for enhancer-trap detection and might account for a considerable fraction of the loci identified if they exist in similar numbers in Drosophila.

VI.3 Expression of *wingless* and *engrailed* in duplicating leg discs

I have shown that both *wingless*, as demonstrated by the expression of the *wg-lacZ* enhancer-trap, and *engrailed*, become ectopically expressed in or near the location of the blastema in 3/4L+EK fragments as determined by Abbott et al. (1981) and by Kiehle and Schubiger (1985). While ectopic *wingless* expression was found to occur in virtually all fragments cultured for at least one day, it did not resolve to an discrete ectopic patch in all fragments cultured longer. The group of fragments where *wingless* expression did not resolve into two discrete patches may correspond to fragments may correspond to those implants which would partially or completely fail to duplicate. Abbott et al. (1981) show that a very high frequency of 3/4L +EK fragments cultured for four days at 25°C duplicate structures from all four sectors (D'=84%, C'=80%, B'=68%, A'=34%, n=98) (see Figure 32). Duplication of D' would imply novel expression of *wingless* in the blastema. In the sample shown in Table 14, 100% of the fragments cultured for 3 days or longer had some expression of *wingless* at the site of the wound (Type III or Type IV fragments), thus the correspondence is quite good. Duplication of C' implies a duplicated posterior compartment and therefore, ectopic expression of *engrailed* would be expected in our fragments. The proportion of fragments where ectopic *engrailed* was observed was lower in our sample (4/16 fragments cultured 3 to 7 days). This may have been due to several factors. In this study, hosts with disc implants were raised at 22°C and the lower temperature may have decreased the frequency of duplications. As well, the ectopic expression of *engrailed* was in general more difficult to detect in fragments stained for both *wingless* and *engrailed*, so it may have not been detected in all cases. Indeed, in the sample of 3/4L+EK fragments which were stained only with *engrailed*, the incidence of ectopic expression detected was somewhat higher (4/11 fragments cultured for 3 or 4 days). Finally it is possible that the fragments in the other study (Abbott et al., 1981) were able to proceed further in pattern regulation when they were subsequently cultured in a larval host prior to metamorphosis. The fragments in this study were only cultured in an adult host and this study is the first to examine the extent of pattern regulation of fragments cultured in adult hosts only using molecular markers as pattern elements.

It is possible to interpret this data in light of recent studies suggesting that *wingless* may have organizing activity in leg imaginal discs (reviewed by Cohen and Di Nardo, 1993). The organizer is a concept with roots in classical amphibian embryology (reviewed in Slack, 1991). The dorsal marginal zone of amphibian blastula-stage embryos is the site of formation of the primary embryonic axis. It is possible to graft the dorsal marginal region of one blastula to an ectopic site in a host blastula. In these embryos a second embryonic axis forms at the site of the graft. The cells in the graft induce a change to dorsal fate in nearby posterior ventral host mesoderm. Analogously, Struhl and Basler (1993) made randomly located clones of cells in developing leg discs which ectopically expressed the *wingless* protein. They argued that dorsally located clones (i.e remote from the normal ventral domain of *wingless* expression) could induce an ectopic proximal-distal axis containing ventro-lateral structures by exerting a ventralizing influence on neighboring cells. They interpreted this as evidence for an organizer activity. Campbell et al. (1993) have proposed that in leg and wing discs, a restricted region where the domains of *wg*, *dpp*, and the homeobox gene *aristaless (al)* are juxtaposed, represent singularities which act as organizers of the distal-proximal axis. They have determined that when *wg* is ectopically expressed near regions expressing high levels of *dpp*, ectopic patches of al are formed which specify the distal-most parts of the induced ectopic axis. They also showed that ectopic expression of the al cDNA driven by the HSP70 promoter induced ectopic axes from the *wingless* expressing domains of the wing disc. As noted by Campbell, it is especially remarkable that the combination of molecules identified as parts of the putative imaginal disc organizer (a Wnt protein, a homeobox protein and an TGF-β protein) are also apparently involved in the establishment of the organizer in *Xenopus* (Cho et al., 1991;Sokol and Melton, 1992; McMahon and Moon, 1989).

The expression of*dpp* is restricted to a stripe of cells just anterior to the *engrailed* expressing cells in leg discs, with higher levels of expression in the dorsal half of the stripe (where *wg* is not expressed) (Massucci et al., 1990). The *engrailed* gene is thought to be required for the correct spatial regulation of *dpp*, as *dpp* expression extends into the posterior compartment in the *en^1* mutant (Raftery et al., 1991). It is possible that *dpp* would become expressed following *wg* and *en* expression in the blastema as this would represent a new confrontation of *engrailed* expressing and non-expressing cells, which is the usual location of *dpp* expression. If this were the case, a new juxtaposition of *wg* and *dpp* would result. This might subsequently induce the ectopic expression of *al* causing the formation of a new axis, hence the duplication.

The extension of *wingless* expression into the region of the horizontal edge of the wound is remarkable in light of a study by Karpen and Schubiger (1981). They implanted 3/4L+EK fragments into an adult host for one day of culture, recovered the fragment and dissected the partially healed horizontal edge from the rest of the implant and re-implanted these either into a larval host where they differentiated structures from sector D only (see Figure 32) or they cultured the fragment in a second adult host prior to implantation into a larval host. Quite unexpectedly, the implants from the second series were frequently able to regenerate the entire disc pattern. This cannot be accounted for on the basis of the French polar coordinate model (French et al., 1976; Bryant et al., 1981).

The results of Karpen and Schubiger (1981) mean that this blastema has the capability to organize the pattern for an entire disc. This has been further interpreted as meaning that the remaining part of the disc merely restricts the potential of the blastema rather than instructing it (Schubiger and Karpen 1983). If the blastema has organizer activity because of the recruitment of *wingless* expression into cells at the healing wound, then a 3/4L+EK fragment would have two organizing foci which would explain the duplications observed. In the Karpen and Schubiger experiment, the new organizer is removed from the influence of the original *wingless* expression and is then able to organize a single complete pattern. This result would be analogous to the organizer grafts inducing ectopic axes in Xenopus blastulas described previously.

In this context, ability to duplicate becomes the ability of a fragment to produce an ectopic organizer region, and the ability to regenerate becomes the ability of a fragment with no organizer to regenerate one. Thus the blastema of the one-quarter upper medial leg fragment (1/4UM), a regenerating fragment which is complementary to the 3/4L+EK fragment and regenerates frequently, would be predicted to also acquire *wingless* expression in order to establish a novel organizer. I have not tested this prediction of the model.

3/4L+EK fragments were never observed to have ectopic expression of *engrailed* in the disc epithelium before 3 days of culture. It is possible that the *wg* expression in the blastema is necessary to activate *en* expression. This would not be unprecedented as Bejsovic and Martinez-Arias (1991) have demonstrated in temperature shift experiments in a *wg*^{ts} background that the removal and later restoration of *wg* expression causes the loss and restoration of *engrailed* expression. Alternatively, the expression of *engrailed* in the peripodial membrane which overlies the horizontal edge may somehow assist in the reactivation of *en* expression in the blastema perhaps through a process analogous to homeogenetic induction observed in Xenopus (reviewed in Slack, 1991).

VI.4 Regeneration models and gene expression patterns selected by the screen

Although null mutant phenotypes will be necessary to assign definitive functions to individual genes, the preferential recovery in the screen of particular expression classes may provide some useful preliminary insights. Wound healing initiates regeneration by bringing into contact cells from different locations. This is thought to stimulate cell proliferation and the interpolation of intermediate positional values in the regeneration blastema (French et al., 1976).

VI.4.1 Wound healing:

Morphological evidence has been obtained during wound healing for novel disc-cell behaviors such as motility, extension of pseudopodia and establishment of new contacts (Reinhardt et al., 1977; Reinhardt and Bryant, 1981). About a third of the insertions expressed in discs only after wounding define functions expressed normally in the embryonic CNS and PNS (Table 4). Other insertions with putative neurogenic primary functions are those expressed in the eye disc posterior to the morphogenetic furrow. This is where assembly of ommatidia occurs, a process involving cell rearrangements and formation of specific contacts, cell-communication leading to cell determination, and some cell death (Tomlinson, 1985; Cagan and Ready, 1989). I speculate that neurogenic functions may be recruited in regeneration to mediate wound healing and cell-communication.

VI.4.2 Establishment of field polarity

The selected insertions are enriched for spatially patterned expression in subsets of follicle cells (26% as compared to 9% among the unselected insertions of Grossniklaus et al., 1989, $\chi^2=5.3$, 1 d.f., $0.05>p>0.01$) and five out of seven ectopically expressed compartment boundary lines are also expressed in either follicle cells or germarium. Spatially patterned gene expression in the follicle cells helps determine the polarity of the embryo (Manseau and Schupbach, 1989; Ruohola et al., 1991) so the present results suggest that there might be common steps in the pathways for polarity specification in the regeneration blastema and in oogenesis. Other insertions are expressed in the embryonic germ band which includes the precursors of the adult epidermal cell-lineage. For example, H-15 is expressed in a segmental repeat pattern in the lateral epidermis which could indicate a role in compartmental subdivision of embryonic segments.

VI.4.3 Positional specification and compartment boundaries

Fifteen of sixteen compartment boundary lines in the entire sample of 826 insertions were either class I or II lines, and all seven lines tested were expressed ectopically in disc fragments. The possible significance of the expression of a/p boundary line insertions in the regeneration blastema is two-fold. 1) The expression of these lines in cultured fragments was found to occur only when cells of the anterior and posterior compartments came into contact as a result of wound healing. This demonstrates gene expression dependent on the original position of the cells in the blastema. It is a necessary feature of many explanations of regulative behaviour of imaginal disc fragments that the original location of cells confronted at wound healing determines the extent of intercalation (French et al., 1976; Bryant et al., 1981; Meinhardt, 1983; Schubiger and Karpen, 1983). 2) It supports the idea that compartment boundaries act as local organizers for the global specification of positional values (Meinhardt, 1983; Gelbart, 1989; Ingham and Martinez-Arias, 1992). The simple geometry of compartmentalization (Garcia-Bellido et al., 1973) would lend itself to specification of an orthogonal system of spatial coordinates, and it has been demonstrated that cooperative involvement of cells from the anterior and posterior compartments is required for distal regeneration (Schubiger and Schubiger, 1978; Karlsson, 1980). Genes expressed in compartmentally restricted patterns might provide the initial positional signals for the respecification of pattern (Meinhardt, 1983). The early re-establishment of compartment boundaries during regeneration has been observed in studies of somatic clones in regenerating imaginal discs (Szabad et al., 1979; Girton and Russell, 1981; Abbott et al., 1981). Bryant and Fraser (1988) found that intercalary cell division in cultured wing discs did not begin until 1-2 days of culture.

In the present study, the re-expression of insertions at the a/p boundary occurred by 24 hours of *in vivo* culture. Thus the re-establishment of compartment boundary gene expression, which probably indicates the re-establishment of the compartment boundary, must occur either before or near the beginning of intercalalary cell division in the regeneration blastema. This means that the first pattern elements to be re-established may be the compartment boundaries. This would not necessarily be predicted from a polar co-ordinate model of imaginal disc regeneration. In a boundary model for positional information, the early re-establishment of organizer activity at a new compartment boundary would be a necessary pre-condition that drives subsequent patterning events in regeneration or duplication

VI.4.4 Blastema formation and the specification of ectopic axes.

Several lines of evidence presented above suggest that the insertions selected in this screen are expressed co-incidentally with the processes of wound healing and blastema formation. Considering the enhancer-trap expression patterns as markers for these events, a hierarchy of gene regulatory events during pattern regulation leading to a duplication event can be postulated. This hierarchy is based on comparisons of the initiation of expression of the enhancer traps selected in this study and the expression of the segment polarity genes *wingless* and *engrailed* in duplications induced either in the cell-lethal background or in cultured 3/4L+EK leg disc fragments.

The ectopic expression of the regeneration induced enhancer-traps was detectable by the end of the heat-treatment with few exceptions. This was found to precede the ectopic expression of *wg* in heat-treated *su(f)* discs which was not detected until 6 hours later . The enhancer-traps were expressed in almost all *su(f)* treated discs and in many cases (i.e. Figure 5) they were expressed in distributions consistent with previously reported *su(f)* induced cell death patterns (Clark and Russell 1977). The changes in *wg-lacZ* expression were more limited in distribution and ectopic patches of *wingless* were seen in a smaller proportion of treated discs consistent with the frequency of duplications induced (Girton and Russell, 1980). The expression of *wg-lacZ* in the *su(f)* and 3/4L+EK leg discs was remarkably similar, supporting the idea that by the stage of *wingless* ectopic expression, the events that lead to duplication in these two systems are the same. The expression in both cases was found to stretch or spread along the lateral circumference of the anterior compartment which eventually resolved into a discrete patch of *wingless* expression. The insertion G-45 is expressed at the horizontal edge in 3/4L+EK fragments after one day in culture. This precedes the earliest ectopic expression of *wg-lacZ* in the wound.

Thus the available evidence shows that the enhancer-traps I selected in my screen are mostly expressed prior to the ectopic activation of *wg-lacZ* in both experimental systems (Tables 12, 13, 14 and Figure 36). It is possible, therefore, that the genes these insertions identify may mediate the processes of wound healing and exchange of positional signals across the healed wound that ultimately determines whether a given blastema will turn on the expression of *wingless* and thus give rise to a secondary axis (duplication). Given the potential role of *wingless* in axis specification it will be crucial to determine whether the ectopic expression of *wingless* is dependent in some fashion on the function of the genes reported by these enhancer-traps.

VI.5 Future directions

This thesis has presented the results of an investigation of the genetic control of imaginal disc regeneration. I have definitively identified a small number of genes expressed in patterns consistent with a significant involvement in wound healing and blastema formation and I have shown that large numbers of these genes may exist. Ultimately, it will be necessary to test how the functions encoded by these genes interfere with the regeneration process by mutational analysis. If mutants can eventually be isolated, an efficient way of screening for function during regeneration would be to make use of the high efficiency induction of somatic clones using the FLP recombinase in a *su(f)* cell lethal background (Golic and Linquist, 1989; Xu and Rubin 1993).

It is remarkable that the formal rules devised to account for regeneration in insect epithelial systems also appear to work in the regenerating vertebrate limb (French et al., 1976; reviewed by Stocum, 1991). In addition to the suggested conservation of genes controlling the organization of pattern in Drosophila imaginal discs and Xenopus embryos stated above, it was recently shown that certain of the HOX-complex genes, highly conserved vertebrate homologues of the *Drosophila* homeotic selector gene complexes, are spatially regulated by positional signals in the developing limb bud (Izpisua-Belmonde, et al., 1991) and a homeobox gene has been found to be redeployed in regenerating amphibian limbs (Brown and Brockes, 1991). In view of this, it will be of great interest to determine if the genes identified in this study also have functional homologues in other phyla.

VII. Bibliography

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